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A STUDY OF SOME ASPECTS OF THE OXIDATIVE
DETERIORATION OF EDIBLE FATS, ESPECIALLY
MILK FAT

by

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A THESIS

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ABSTRACT

This study was undertaken to evaluate and improve some of the analytical methods used for the characterization of fat autoxidation, and to test the effect of selected antioxidants on milk fat, cottonseed oil, and lard autoxidation.

An improved polarographic method was found for the determination of copper and iron in fats at levels of 0.01 p.p.m. or higher.

During the customary conditions of the 2-thiobarbituric test the possibility of carbonyl losses was proven. When the condensation was carried out at low temperature or at constant volume, the losses could be avoided. Acetaldehyde and crotonaldehyde gave pigments with 2-thiobarbituric acid that had an absorption maximum at 525 m μ . In the presence of iron (III) ions crotonaldehyde yielded a pigment that did not follow Beer's Law. Molecular weight determinations, elemental analyses, and infrared spectra showed that the malondialdehyde-2-thiobarbituric acid pigment can be a small integer multiple of a basic structure, the size depending on the medium.

The activation energies, turnover numbers, and temperature coefficients of the lipoxidase-linoleic and linolenic acid reactions were determined in the presence of 50 per cent

competitive inhibitor. It was concluded that lipoxidase could initiate autoxidation.

Some destruction of unsaturated carbonyls was observed during the thermal oxidation of a butter concentrate in the presence of different oxygen concentration under ultraviolet light.

Fresh milk fat samples were found to contain copper and iron even after hot water washing, and they contained iodometrically but not polarographically measurable peroxides.

In accelerated tests, ascorbyl palmitate was found to be an effective antioxidant for milk fat and cetyl gallate for lard and cottonseed oil.

Accelerated oxidation changed the index of refraction of lard and cottonseed oil, but not that of milk fat. Steam volatile distillates from these fats contained more unsaturated than saturated carbonyls, while in the fats the reverse was true with the exception of cottonseed oil. The exit gases from lard and cottonseed oil contained carbon dioxide and those from milk fat and lard short chain fatty acids. In the polarograms of autoxidized fats the prewave was assigned to peracids. The mangan (II) wave overlapped with that of 2-unsaturated aldehydes in polarographic peroxide determinations.

It was found that high temperature (100°C) promotes the formation of free fatty acids of autoxidative origin. The level of these acids is a measure of the oxidative changes.

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A STUDY OF SOME ASPECTS OF THE OXIDATIVE DETERIORATION OF EDIBLE FATS, ESPECIALLY MILK FAT

GENERAL INTRODUCTION

The so called cardboard, tallowy, fishy, oily, metallic, and cucumber flavours of edible fats are caused by various carbonyl compounds which are formed by the breakdown of the primarily produced hydroperoxides (24). These off-flavours develop sooner or later at a wide range of keeping temperatures provided oxygen is available. The process of oxidation is autocatalytic because part of the formed hydroperoxides re-enters the oxidation cycle by the means of a free radical chain reaction (10), and it is catalyzed by ultraviolet light, traces of copper and iron, and various pigments such as the carotenoids and chlorophyll. The human taste buds are highly sensitive to the above mentioned oxidized flavours (48, 66). Most of these flavours are disliked, and such deteriorated foodstuffs are rejected by the discriminating consumer. Moreover, not only the full, pleasing, and rich flavour of the fat is impaired through oxidative changes, but the nutritive value of the food is lowered, because of the destruction of most of the fat soluble vitamins, particularly vitamins E and A, and part of the

essential fatty acids (35, 25, 4) . Furthermore, there is some evidence to support the view that oxidized fats are toxic to living organisms (75, 65, 58) . Therefore, the problem of keeping fat containing foods free from oxidative deterioration has received a great deal of attention.

As the problem stands now, the systematic and successful counteraction of oxidative rancidity is very difficult mainly because of the inadequate knowledge of the phenomenon involved. The approach in this study is one of an initial fundamental investigation of the sensitivity, range of applicability, and mode of action of several tests used for the characterization of fat autoxidation. The importance of the estimation of the levels of trace metals, peroxides, carbonyls, free fatty acids, and their correlation is especially emphasized. The possibility of enzymatic free radical initiation is also studied, besides the effect of temperature and ultraviolet light on the stability of peroxides and carbonyls. Accelerated tests are conducted in order to appraise the effectiveness of selected antioxidants and the changes brought about in autoxidizing milk fat, cottonseed oil, and lard.

A POLAROGRAPHIC DETERMINATION OF COPPER AND IRON IN FATS

INTRODUCTION

Traces of metals, especially copper and iron, are powerful catalysts of fat oxidation. The metal content of commercial fats and oils is usually in the 0.1 - 1.0 p.p.m. range (63). At these levels the rate of free radical initiation is directly proportional to the concentration of the catalyst (15). Therefore, the accurate measurement of the level of these contaminants is of paramount importance from the point of view of predicting the keeping quality of fats. There are several colorimetric methods for the estimation of small amounts of copper and iron in fats. Such a method is that of Perrin et al. (68). Their technique is applicable not only to dairy products, but also to other fatty or proteinaceous materials. However, the development of the colours is long and laborious, and the determination tends to yield high results. A polarographic technique for the determination of iron, copper, and nickel in lard samples weighing up to 200 grams has been worked out by Lupton et al. (56). It is based on the instantaneous decomposition of lard introduced drop-wise into a hot

crucible. This step is followed by ashing and a potassium hydrogen sulphate fusion of the residue. The metals are determined polarographically using the above mentioned salt as supporting electrolyte. The separation of the copper and iron waves in this medium, however, is not entirely satisfactory. Poor separation of the waves is especially pronounced where the iron to copper ratio is large. The advantage of this method is the possibility of using large samples when the copper concentration is lower than 0.01 p.p.m. and the iron content is below 0.5 p.p.m. However, the gain in sensitivity is counteracted by loss of accuracy, because the escaping vapours, smoke, and aerosol of lard during ashing can remove particles of metal rich residue that has already been collected.

It was decided that a procedure should be worked out which permits the ashing of fat samples without losses, and which permits the utilization of the basic virtues of polarography: simplicity of measurement, the possibility of the simultaneous determination of different solutes in a common solution, and high sensitivity. A supporting electrolyte was sought that yields well defined and well separated polarographic waves for copper and iron.

EXPERIMENTAL

Ashing. Five grams of anhydrous fat are weighed into a large Vycor crucible (volume 80 ml, top outside diameter 65 mm) and 2 grams of ammonium nitrate (Merck, Reagent) and 5 ml of concentrated sulfuric acid (Mallinckrodt, Analytical Reagent, Sp. Gr. 1.82/25°C) are added. The crucible is placed on a hot plate at 110°C under a fume hood. After 15 minutes the temperature is increased to 250°C and held at that point until the foaming stops. The temperature is then increased to 350°C and kept there until the foaming and fuming ceases and a tarry substance is obtained. This mass is heated further on a 600°C hot plate. A solid, carbonaceous residue is produced. If too much carbon is in the residue, the above treatment is repeated with 1 gm ammonium nitrate and 2 ml sulfuric acid. The ashing is completed in a 600°C muffle furnace. Finally, the crucible and its contents are cooled to below 100°C and the ash is covered with 50 mg of potassium bicarbonate (Merck, Reagent). The mixture is then fused first on a hot plate at 250°C followed by 20 minutes in the muffle furnace at 600°C.

A blank is prepared with the same amount of chemicals as the sample.

Solutions. Kolthoff and Lingane (43) suggested on the basis of the work done by Lingane (51) and Meites (60) that copper and iron could be determined in a common solution polarographically, using 0.5 molar sodium tartrate solution at pH 12. The following up of this idea led to satisfactory results.

(1) Supporting electrolyte stock solution is 0.56 normal sodium-potassium tartrate (British Drug Houses, Analytical Reagent). The solution should be neutral.

(2) The fused ash in the crucible is carefully dissolved in 1 ml of 5 N hydrochloric acid (Fisher, Reagent Chemical). Four ml of 1.5 N potassium hydroxide (Merck, Reagent) is added. The solution is quantitatively transferred to a 50 ml volumetric flask using 0.56 N sodium-potassium tartrate as a rinse. The volume is made up to the mark with the same solution. The pH of this solution should be close to 12.

(3) Standard copper and iron solutions. Twenty mg of electrolytic copper (Fisher, Certified) and 20 mg of pure iron wire (Fisher, Certified) are dissolved in a mixture of 5 ml of 5 N nitric acid (Merck, Reagent) and 10 ml of 5 N sulfuric acid by gentle heating. The heating is continued until the nitric acid is completely displaced. The solution is transferred to a one liter volumetric flask and made up to the mark with demineralized distilled water. A three ml aliquot is transferred

to a 50 ml volumetric flask, 2 ml of 1 N potassium hydroxide is added and the solution is made up to mark with 0.56 N tartrate solution. This solution contains 0.060 milligram iron and copper.

Recording the polarogram. Approximately 15 ml portions of the iron and copper solutions are deaerated, and under anaerobic conditions and at 25°C the cathodic wave is obtained for the 0-2 volt range. The residual current is obtained on the blank. The open circuit electrode data is as follows. The drop time is between 4 and 5 seconds; the mercury flow rate is 2 mg/sec; total cell resistance is 660 ohms. Required current sensitivity is 0.001-0.006 μ A per millimeter of chart. No maximum suppressor is required, and none should be present as it would obliterate the copper wave.

A Sargent Model XV Recording Polarograph with a Micro Range Extender was used for recording the polarograms. In the absence of RC damping this instrument registers the maximum current during the life of the mercury drop. Therefore, the diffusion current is obtained between the appropriate two tops of the oscillatory trace at the half wave potential. The corresponding form of the Ilkovic equation is as follows (43).

$$i_{\max.} = 706 n D^{1/2} m^{2/3} t_{\max.}^{1/6} C$$

where:

- $i_{\max.}$ = the maximum diffusion current,
in microamperes
- n = number of electrons transferred
per reduced molecule
- D = diffusion coefficient of electro-
reducible species in square centi-
meters per second
- m = flowrate of mercury in milligrams
per second
- $t_{\max.}$ = maximum drop time of mercury in
seconds
- C = concentration of the reducible species
in millimoles per liter

All glassware used in this determination should be washed in hot detergent solution and rinsed thoroughly with demineralized distilled water.

RESULTS AND DISCUSSION

The polarograms of cupric and ferric ions in the common tartrate medium at pH 12 are composed of three well developed and well separated waves (Fig. 1). Twelve randomly selected polarograms were evaluated for the computation of the values shown in Table 1. The half wave potentials are corrected for

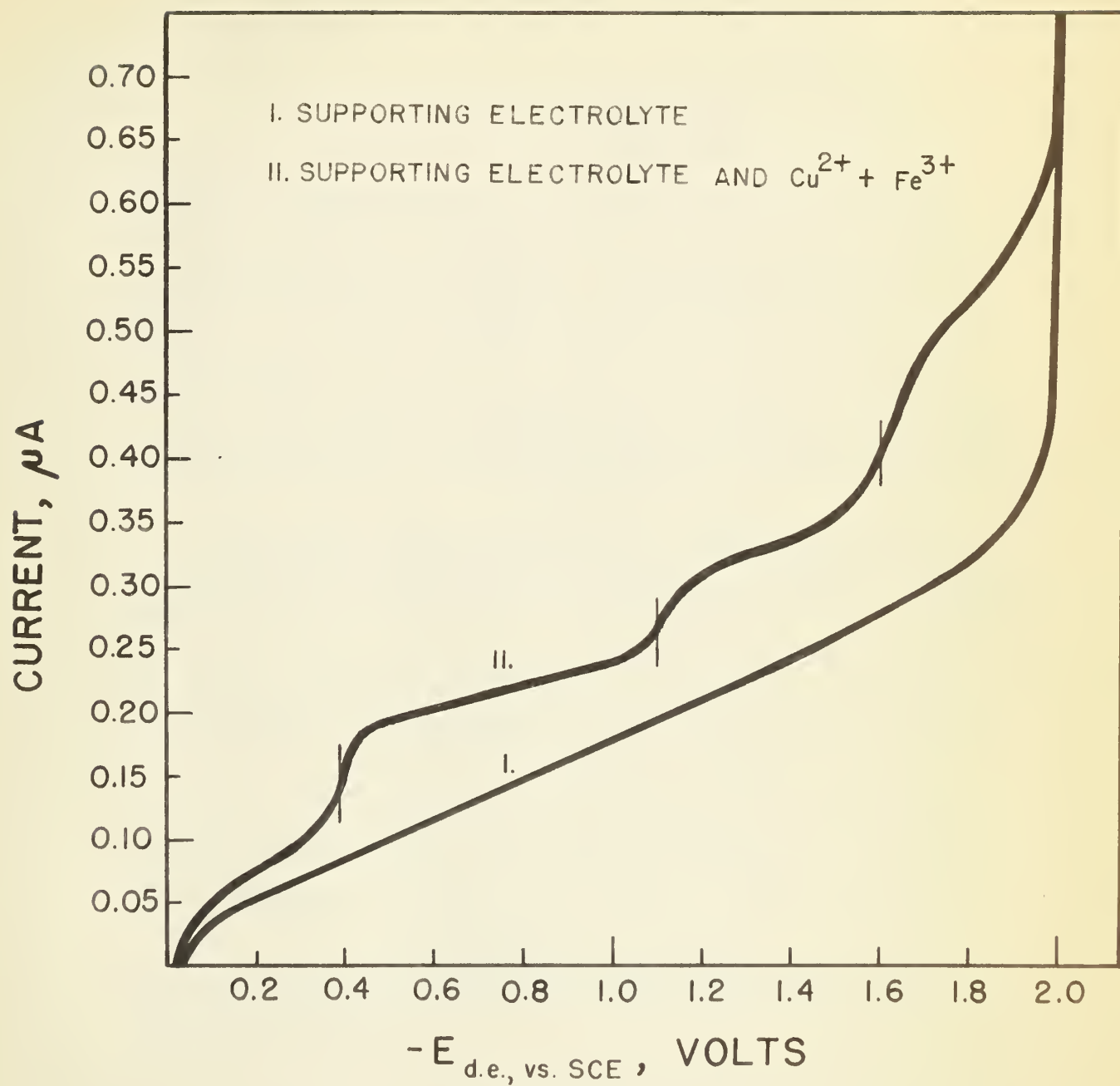


Fig. I. Polarogram of $10^{-5} M Cu^{2+}$ and Fe^{3+} ions

IR drop, and they are reported with respect to the potential of saturated calomel electrode (SCE). The values of \underline{n} were calculated from

$$n = 0.0591 \frac{|\log(i/i_d - i)|}{|E_{1/2} - E|},$$

and \bar{n} was computed from all \underline{n} values that correspond to the same half wave potential waves. The average values of $\underline{E}_{1/2}$, $\underline{i}_{\max.}$, and \underline{n} are listed together with the standard error of the mean. The small standard error of \bar{n} for the three waves shows that the $\underline{E}_{d.e.}$ versus $\underline{\log(i/i_d - i)}$ plot is essentially linear.

With these values it is possible to relate $\underline{i}_{\max.}$ to the concentration of copper and iron without using standard solutions. However, because of the large deviation of the \bar{n} values from small integers, which might point to an irreversible electrode reaction, it is advisable to use the above standard solutions. In this case the number of milligrams of copper or iron in a volume of 50 ml is $\underline{0.060 (i_x/i_s)}$, where $\underline{i_x}$ is the maximum diffusion current of the unknown metal concentration and $\underline{i_s}$ is that of the standard.

Table 1. Polarographic constants of cupric and ferric
ions in 0.5 molar sodium-potassium tartrate
at pH 12 and 25°C

Wave	Ion	Conc. mM/l	$-\bar{E}_{1/2} \pm s$ volts vs. SCE	$\bar{i}_{\max.} \pm s$ μA	$\bar{n} \pm s$	$\frac{i_{\max.}}{C_m^{2/3} t^{1/6}}$
1 st	Cu^{2+}	.01692	.390 \pm .004	.080 \pm .003	1.2 \pm .1	2.32
2 nd	Fe^{3+}	.11509	1.170 \pm .006	.399 \pm .008	.76 \pm .04	1.70
3 rd	Fe^{3+}	.11509	1.600 \pm .000	.54 \pm .01	.74 \pm .04	2.30

Ashing m grams of fat, the corresponding concentration of copper and iron in p.p.m. is

$$(60/m) \times (i_x/i_s) .$$

The good recoveries of copper and iron, (Table 2) , obtained by ashing known amounts of cupric and ferric sulfates with 4 grams of palmitic acid, using palmitic acid with no iron and copper added as blank, indicates that the ashing procedure is satisfactory and that the observed diffusion currents are proportional to the concentration.

Table 2. Recoveries of copper and iron in a polarographic determination

Sample No.	Quantity in sample, mg		Recovery, %	
	Copper	Iron	Copper	Iron
1	.01	.01	98.0	95.3
2	.01	.10	98.4	97.1
3	.50	.10	95.1	96.8
4	.50	.50	98.5	94.6
5	.50	1.00	99.1	93.7
6	1.00	1.00	96.3	94.0

SOME OBSERVATIONS ON THE 2-THIOBARBITURIC ACID TEST

INTRODUCTION

The 2-thiobarbituric acid (2-TBA) test is concerned with the estimation of the state of oxidation of lipids through the measurement of the intensity of a red colour in a suitable solvent. The pigment is obtained as the result of a reaction between 2-TBA and malonic dialdehyde, besides other aliphatic aldehydes, in a strongly acid medium on heating.

Carbonyls are formed in autoxidizing fats mainly as the result of the decomposition of the hydroperoxides of unsaturated fatty acids; some of these carbonyls are responsible for the oxidized flavour of lipids and lipid containing foods. The use of the 2-TBA test for the food industry would be to measure off-flavour causing carbonyls at all concentration levels but possibly well before these are organoleptically noticeable, or to measure a carbonyl concentration that gives an exact description of the level of deterioration of the oxidized lipid regardless of its overall composition and autoxidizing environment. The 2-TBA test should be also useful for comparison of the stage of oxidation of lipid samples of the same origin.

These requirements have never been completely realized.

Although there is a high correlation among the results of the 2-TBA test, peroxide values, and taste panel scores of a given sample, the 2-TBA test reveals very little about the processes of autoxidation partly because the kinds and combinations of carbonyls that react with 2-TBA have never been conclusively established, and partly because the exact nature of the formation of carbonyls from hydroperoxides is not known. Besides these inherent difficulties, the conditions for the completion of the reaction between 2-TBA and carbonyls permit the occurrence of undesirable side reactions of unknown origin that cause some non-specific absorption due to degradation products of unreacted 2-TBA (87).

It is obvious then that the study of the mechanism of the 2-TBA reaction and the conditions of the test are desirable if the major virtue of the test, its relatively high sensitivity, is to be utilized.

REVIEW OF LITERATURE

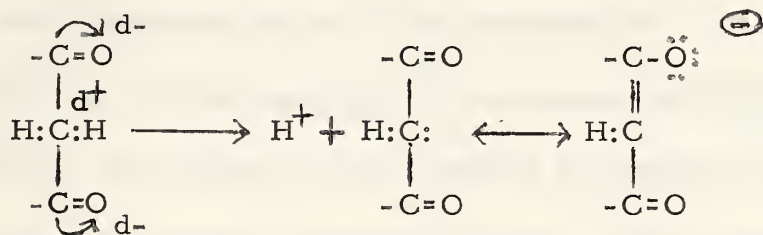
As a result of the investigation of the properties of barbituric acid derivatives - which was started by the synthesis of barbituric acid by Baeyer in 1863, and by the discovery of

the hypnotic action of barbiturates - 2-TBA was synthesized by Michael in 1887. The explicit directions for the synthesis are given by Gabriel and Colman (26). Utilizing the reactivity of the methylene group of 2-TBA and the aldehyde group of furfural, Dox and Plaisance successfully determined pentosans after converting them to furfural by an acid treatment (20). The same workers determined several aromatic aldehydes by the same technique (21). In 1944 Kohn and Liversedge (42) observed that aerobically respiring rat brain tissue suspensions yielded a compound that gave an orange-red colour with 2-TBA. The amount of this metabolite was increased if oxygen was used for respiration instead of air. It was deduced that this compound "B" involved is an aldehyde. Bernheim et al. (5) came to the conclusion that the reacting compound in the above mentioned experiment is of lipid origin and that it must be an aldehyde of a three carbon atom fragment of linolenic acid. Patton and Kurtz (67) proposed that the 2-TBA test could be used for the detection of milk fat oxidation, and they emphasized the potentiality of the test for the detection of the oxidative deterioration of other fat containing foods as well. They tentatively identified the aldehyde responsible for the reaction with 2-TBA as malonic dialdehyde by comparing the visible absorption spectrum of the colour obtained as the result

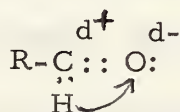
of the reaction between milk fat and 2-TBA with that obtained from the condensation of this reagent and malonic dialdehyde. The two absorption spectra were identical. The sensitivity of the test was found to be less than 1 p.p.m. of malonic dialdehyde. Biggs and Bryant (7) tested the effect of oxygen, time, and pH on the reliability of the 2-TBA test when used for dairy products. Sinnhuber and Yu (80) put the 2-TBA test on a more quantitative basis when they introduced the use of 1, 1, 3, 3 = tetraethoxy propane, an acetal that yields malonic dialdehyde on hydrolysis, as a standard compound for obtaining extinction curves for the malonic dialdehyde 2-TBA complex, and they proposed the use of "TBA numbers", meaning mg malonic dialdehyde per kg sample, for the comparison of the state of oxidation of different samples.

The properties of 2-TBA can be considered from two points of view. To begin with, almost all 2-TBA derivatives are coloured because they contain chromophoric groups such as $=CO$, $=CS$, $-CH=CH-$, and the following auxochromes $-OH$, $-NH-$, $-SH$. This property makes it possible to determine these derivatives colorimetrically. On the other hand, the reactivity of 2-TBA is due largely to its number 5 carbon atom which is activated by the electron releasing substituents 2,4,6 of the pyrimidine ring. The molecule can lose two protons

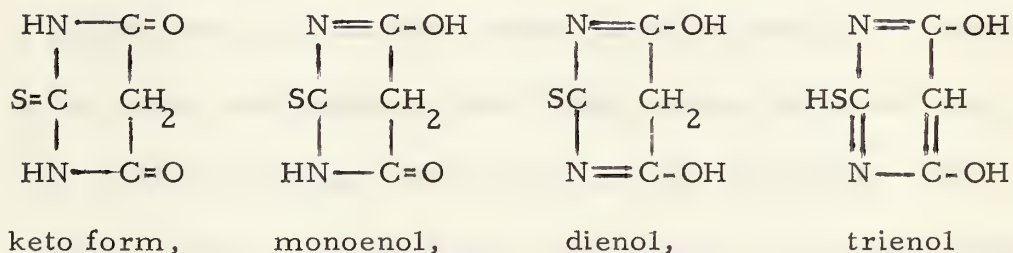
relatively easily because the carbanion formed is resonance stabilized, as it is shown by the following formulas.



Therefore, the number 5 carbon atom can undergo an electrophilic attack by such groups as the partial positive charge bearing carbon of carbonyls.



2-TBA can exist in four tautomeric forms.



The relative amounts of these tautomers are pH dependent, but the equilibria have not been studied on a quantitative basis. The carbonyl - 2-TBA reaction requires a pH lower than 2.

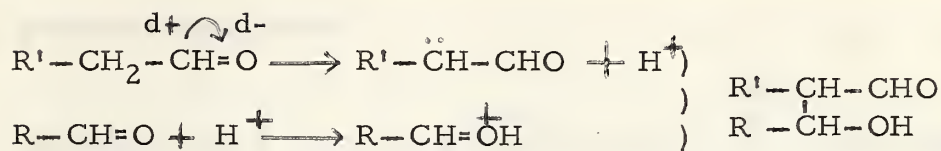
The electron deficient pyrimidine ring is quite stable toward oxidation. Desulfurization of 2-TBA, however, can be easily brought about by H_2O_2 . Hydrochloric or other strong

acids on heating can replace the -SH group on the trienol form by -OH; barbituric acid does not react with malondialdehyde. In a thorough investigation of the behaviour of 2-TBA during acid heating, Tarladgis et al. (87) demonstrated the instability of 2-TBA. According to these authors the reagent is partly hydrolyzed to malonic acid and thiourea. These compounds in turn decompose to CH_3COOH , CO_2 , and CO_2 , NH_3 , H_2S respectively. In contrast to this, an oxidizing agent such as H_2O_2 or R-OOH might give rise to the formation of disulfides, sulfoxides, sulfones, and alloxan. The curious thing is that these workers could not isolate the breakdown products. At any rate, it has been confirmed that during acid-heat treatment of 2-TBA changes take place which result in absorption maxima at the same wavelengths as the 2-TBA-malondialdehyde complex. The evidence presented by Tarladgis et al. (87) points to the special importance of the proper preparation of the blank solution. Moreover, it is obvious that although the 2-TBA test is easily carried out, the interpretation of the results is rather difficult.

It is generally accepted that in the case of oxidized lipids the 2-TBA reactive carbonyl is malonic dialdehyde. It will be demonstrated that although the 2-TBA test is most sensitive for this aldehyde, other aliphatic aldehydes give a similar colour reaction. Malonaldehyde can be involved in the following

reactions with other components of autoxidizing fats.

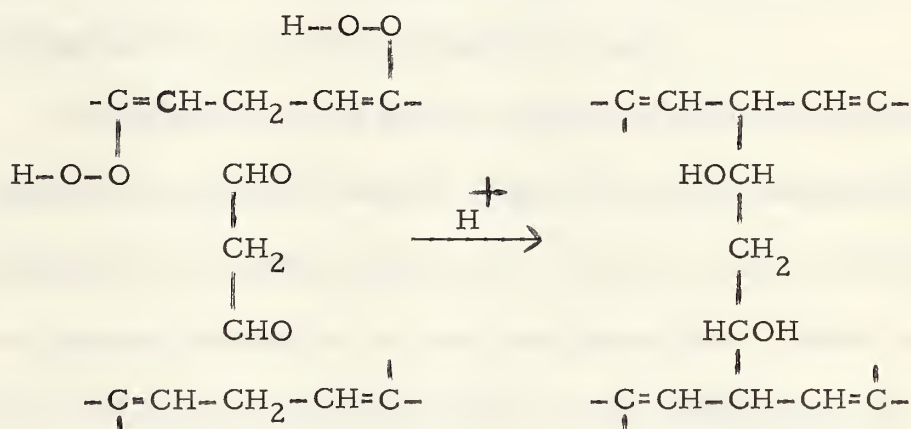
(a) Acid catalyzed condensation of carbonyls:



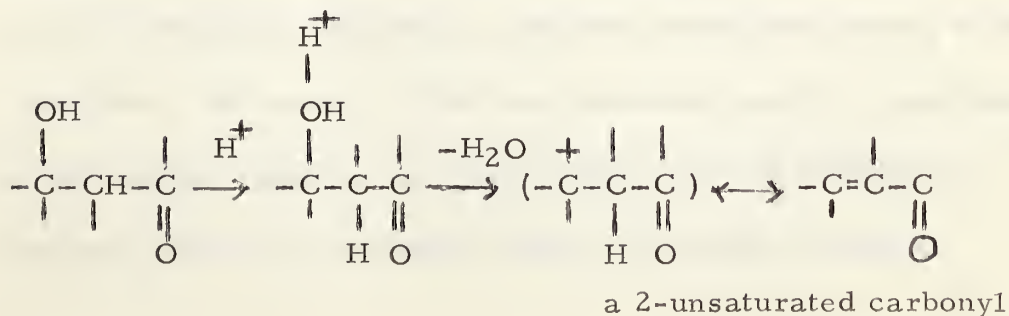
an aldol type compound

Because an aldehyde group remains intact in such a bimolecular reaction, polymerization can go on to attain high molecular weight compounds.

(b) Malondialdehyde can be tied up between two adjacent unsaturated chains:



(c) Aldol type compounds are easily inter-convertible in acid catalyzed dehydration to 2-unsaturated carbonyls:



1. $\frac{1}{x^2} = x^{-2}$ and $\frac{d}{dx} x^{-2} = -2x^{-3} = -\frac{2}{x^3}$

2. $\frac{d}{dx} \frac{1}{x^3} = \frac{d}{dx} x^{-3} = -3x^{-4} = -\frac{3}{x^4}$

$$\frac{d}{dx} \left(\frac{1}{x^2} + \frac{1}{x^3} \right) = -\frac{2}{x^3} - \frac{3}{x^4}$$

Answer: $-\frac{2}{x^3} - \frac{3}{x^4}$

3. $\frac{d}{dx} \left(\frac{1}{x^2} - \frac{1}{x^3} \right) = -\frac{2}{x^3} + \frac{3}{x^4}$

4. $\frac{d}{dx} \left(\frac{1}{x^2} + \frac{1}{x^4} \right) = -\frac{2}{x^3} - \frac{4}{x^5}$

5. $\frac{d}{dx} \left(\frac{1}{x^2} - \frac{1}{x^4} \right) = -\frac{2}{x^3} + \frac{4}{x^5}$

6. $\frac{d}{dx} \left(\frac{1}{x^2} + \frac{1}{x^5} \right) = -\frac{2}{x^3} - \frac{5}{x^6}$

7. $\frac{d}{dx} \left(\frac{1}{x^2} - \frac{1}{x^5} \right) = -\frac{2}{x^3} + \frac{5}{x^6}$

8. $\frac{d}{dx} \left(\frac{1}{x^2} + \frac{1}{x^6} \right) = -\frac{2}{x^3} - \frac{6}{x^7}$

9. $\frac{d}{dx} \left(\frac{1}{x^2} - \frac{1}{x^6} \right) = -\frac{2}{x^3} + \frac{6}{x^7}$

10. $\frac{d}{dx} \left(\frac{1}{x^2} + \frac{1}{x^7} \right) = -\frac{2}{x^3} - \frac{7}{x^8}$

11. $\frac{d}{dx} \left(\frac{1}{x^2} - \frac{1}{x^7} \right) = -\frac{2}{x^3} + \frac{7}{x^8}$

12. $\frac{d}{dx} \left(\frac{1}{x^2} + \frac{1}{x^8} \right) = -\frac{2}{x^3} - \frac{8}{x^9}$

13. $\frac{d}{dx} \left(\frac{1}{x^2} - \frac{1}{x^8} \right) = -\frac{2}{x^3} + \frac{8}{x^9}$

14. $\frac{d}{dx} \left(\frac{1}{x^2} + \frac{1}{x^9} \right) = -\frac{2}{x^3} - \frac{9}{x^{10}}$

15. $\frac{d}{dx} \left(\frac{1}{x^2} - \frac{1}{x^9} \right) = -\frac{2}{x^3} + \frac{9}{x^{10}}$

16. $\frac{d}{dx} \left(\frac{1}{x^2} + \frac{1}{x^{10}} \right) = -\frac{2}{x^3} - \frac{10}{x^{11}}$

17. $\frac{d}{dx} \left(\frac{1}{x^2} - \frac{1}{x^{10}} \right) = -\frac{2}{x^3} + \frac{10}{x^{11}}$

18. $\frac{d}{dx} \left(\frac{1}{x^2} + \frac{1}{x^{11}} \right) = -\frac{2}{x^3} - \frac{11}{x^{12}}$

19. $\frac{d}{dx} \left(\frac{1}{x^2} - \frac{1}{x^{11}} \right) = -\frac{2}{x^3} + \frac{11}{x^{12}}$

20. $\frac{d}{dx} \left(\frac{1}{x^2} + \frac{1}{x^{12}} \right) = -\frac{2}{x^3} - \frac{12}{x^{13}}$

21. $\frac{d}{dx} \left(\frac{1}{x^2} - \frac{1}{x^{12}} \right) = -\frac{2}{x^3} + \frac{12}{x^{13}}$

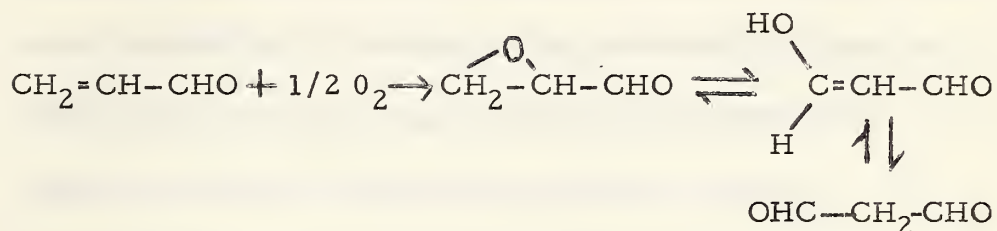
22. $\frac{d}{dx} \left(\frac{1}{x^2} + \frac{1}{x^{13}} \right) = -\frac{2}{x^3} - \frac{13}{x^{14}}$

23. $\frac{d}{dx} \left(\frac{1}{x^2} - \frac{1}{x^{13}} \right) = -\frac{2}{x^3} + \frac{13}{x^{14}}$

24. $\frac{d}{dx} \left(\frac{1}{x^2} + \frac{1}{x^{14}} \right) = -\frac{2}{x^3} - \frac{14}{x^{15}}$

25. $\frac{d}{dx} \left(\frac{1}{x^2} - \frac{1}{x^{14}} \right) = -\frac{2}{x^3} + \frac{14}{x^{15}}$

(d) Malondialdehyde can be formed from a 2-unsaturated aldehyde: acrolein, through free radical oxidation and subsequent rearrangement:



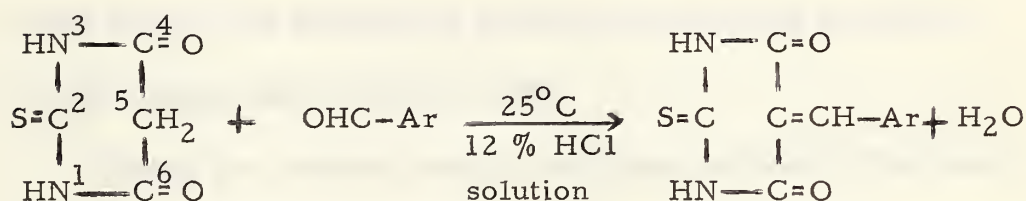
Reactions "a" and "c" are reversible.

The existence of reactions "a-d" in autoxidizing fats has never been demonstrated. Considering the complexity of constituents of autoxidizing lipids, the difficulty involved in elucidating these reactions is quite obvious.

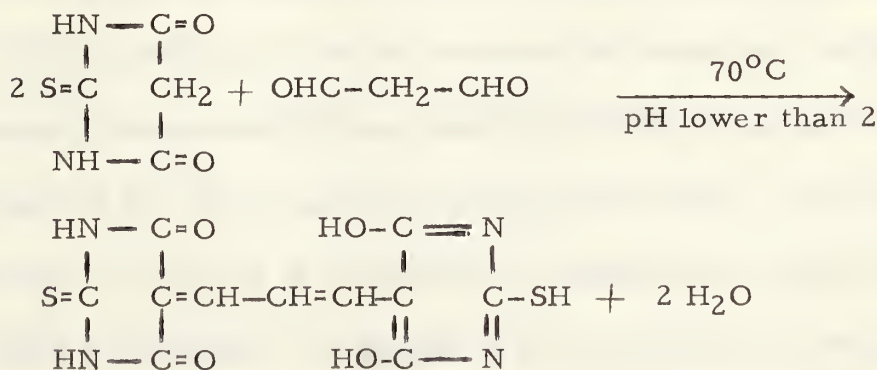
The significance of malonic dialdehyde in oxidizing fats cannot be ascertained, because nobody has ever isolated from oxidized fats or model systems directly or by steam distillation free malonic dialdehyde in significant quantities. Sinnhuber et al. (80) could isolate only about 2% free malondialdehyde if the amount indicated by the 2-TBA test is taken as 100 %. These facts, keeping in mind that the molecular weight of the chromogen was not available, left much doubt concerning the identity of the reactants. Moreover, it has been demonstrated by comparison of absorption spectra, R_f values, and results of elemental analysis that the chromogens resulting from the reactions

between 2-TBA and malondialdehyde or oxidized fish oil or sulphadiazine are identical (80). It is difficult to explain how sulphadiazine, 2-sulphanylamidopyrimidine, could give rise to malondialdehyde. Although the pyrimidine ring can be hydrolyzed, the occurrence of this reaction under the conditions of the 2-TBA test has not been shown.

2-TBA reacts with aryl aldehydes, especially 2-unsaturated aryl aldehydes, according to the following equation (21).



In the case of autoxidized fats, however, the principal reaction thought to be a trimolecular condensation (80) between two molecules of 2-TBA and a molecule of malonic dialdehyde.



The elemental analysis of the crystalline complex (80) suggested the use of the above formula which would correspond to

$C_{11}H_8N_4O_4S_2$ with a molecular weight of 324.35. This value, however, could be n fold, where $n=1,2,3,\dots$, because the molecular weight of the complex was not determined. It will be shown that the estimated molecular weight of the chromogen is not consistent with the above reaction scheme. The complex in solution is of brownish-red colour. According to the theory of complementary colours, it should absorb between 530 and 545 m μ . Once the complex is formed, which requires a pH lower than 2, its absorption spectrum shows only a shift of 15 m μ between pH 1 and 11.4 (76).

There are several ways of carrying out the 2-TBA test. Each of these different procedures is designed to give the most specific test as far as the kind of sample in question is concerned. Generally speaking, these modifications fall into two groups (87). In one group the sample is kept in a highly acid medium with 2-TBA at 60°C for 15 to 30 minutes; the brownish-red pigment formed is extracted with a suitable solvent and the absorbance readings are taken against an appropriate blank. In the other group, the sample is acidified and subsequently subjected to steam distillation. An aliquot of the condensate is treated as above. There are many sub-modifications. The usual variants are the pH of the medium, duration and temperature of heating, and solvents. The explanation of these modifications is that

the TBA numbers obtained in a certain way give a better correlation with taste panel results or peroxide values or total carbonyl contents as measured by the 2,4-dinitro-phenylhydrazine method. The followers of the steam distillation technique claim that the sample is protected from further oxidation during the test, and that only the volatile constituents, which are said to be the principal cause of off-flavours, come in contact with the 2-TBA thus minimizing the danger of formation of complexes that might give rise to non-specific absorption. The existence of these dangers, however, has never been demonstrated. On the other hand, it will be shown that steam distillation or heating the carbonyl-2-TBA solution at 70°C involves the losses of volatile compounds.

EXPERIMENTAL

The characterization of some unknown properties of the 2-TBA test. A steam distillation version of the 2-TBA test according to Täufel and Zimmermann (88) was selected for fat oxidation studies, because of the following reasons. The test is of good reproducibility; it yields blanks with low non specific absorption, and it can be used for the estimation of volatile, unsaturated carbonyls as well as saturated ones. However,

some important aspects of the test were not clarified. The validity of the Beer-Lambert Law was not proven, which made the comparison of the state of oxidation between various samples impossible, and the magnitude of the molar extinction coefficient was not determined. Furthermore, the high volatility of malondialdehyde, saturated, and 2-unsaturated aldehydes necessitated a check of the per cent recoveries during steam distillation, and on the possibility of losses during the early stage of the colour development.

For the verification of the validity of Beer's Law, and the determination of the molar extinction coefficient the following experiment was conducted. A sample of tetraethoxypropane purified by fractional distillation, weighing $2.1884 \text{ g} = 9.9414 \times 10^{-3}$ moles, was hydrolyzed in a solution of 300 ml of water and 1 ml of 3 N hydrochloric acid. The mixture was refluxed for 30 minutes on a boiling water bath in a 1 liter round bottom flask with a ground joint. After cooling to 0°C the contents were quantitatively transferred to a 1 liter volumetric flask, and made up to the mark in two stages, the second at 25°C , with distilled water to give stock solution I. A hundredfold dilution of stock solution I. gave stock solution II., and a thousandfold one stock solution III. From these a set of ten dilutions were made according to Table 3. Aliquots of 5 ml of solutions 1-10

Table 3. Dilution of malondialdehyde solutions

Stock solution	ml/ 100 ml	Flask number	Moles/liter in the final solution (25 ml)
III.	1	1	1.99×10^{-8}
III.	2	2	3.98×10^{-8}
III.	4	3	7.95×10^{-8}
III.	8	4	1.59×10^{-7}
III.	16	5	3.18×10^{-7}
III.	32	6	6.36×10^{-7}
II.	6.4	7	1.27×10^{-6}
II.	12.8	8	2.55×10^{-6}
II.	25.6	9	5.09×10^{-6}
II.	51.2	10	1.02×10^{-5}

in triplicate were transferred to 25 ml volumetric flasks and 5 ml of 2-TBA reagent (saturated solution in glacial acetic acid) was added to each. Sets of samples received one of the following treatments. They were heated immediately after the addition of the reagent for 30 minutes in a water bath at 70°C or left standing at room temperature for 4-12 hours and then heated as above. There were sets that received no heating at all. In an equal number of times the flasks were tightly stoppered during the heat treatment. After the development of the colour, the solutions were diluted to 25 ml at 25°C. The absorbancy readings were taken in a Beckman DU Spectrophotometer at 530 mμ in 1 cm cuvettes. Zero absorbancy was set by using a blank that was treated as the samples except that it contained 5 ml distilled water in place of the malondialdehyde solutions. The pH of the final solutions was measured in the manner described in the lipoxidase experiment (Chapter IV). The entire procedure was repeated twice, including the hydrolysis of tetraethoxypropane. Samples 1-3 gave no measurable colours, which sets the limit of sensitivity of the 2-TBA test for malondialdehyde under the conditions described at approximately 10^{-7} moles per liter. Student's t-test applied to the optical density readings of sets of samples undergoing

the various heat treatments gave the results shown in Table 4. A plot of optical density vs. concentration is shown in Fig. 2 and Fig. 3. The corresponding equation was calculated by fitting the points according to the principles of linear regression. It is based only on those sets of determinations that showed no losses according to Table 4. The molar extinction, E , was calculated from the regression coefficient.

$$E \frac{1 \text{ cm}}{530 \text{ m}\mu / .2 \text{ mm}} = 1.24 \times 10^5$$

The average pH with the standard error of the mean was 1.79 ± 0.01 .

The efficiency of malondialdehyde recovery during steam distillation (88) was checked as follows. An appropriate aliquot of solution 8 in Table 3 was transferred to a 500 ml round bottom flask. After adding 75 ml distilled water and 7 ml 3 normal hydrochloric acid, the solution was subjected to steam distillation according to the specifications of Täufel and Zimmermann (88). To approximately 50 ml of condensate collected in a 100 ml volumetric flask in 10 minutes, 10 ml of 2-TBA reagent (saturated solution in glacial acetic acid) was added. The colour was developed by holding the samples at room temperature for four hours and then in a water bath at 70°C for 10 minutes. On cooling to room temperature, the volume was made up to the

Table 4. Comparison of the effect of various heat treatments on the efficiency of the reaction between 2-TBA and malondialdehyde

		Heating only		Holding only		Holding and heating	
		v	p	v	p	v	p
Heating only	v			-	-	-	-
	p			+	+	+	+
Holding only	v	-	+			-	-
	p	-	+			-	-
Holding and heating	v	-	+	-	-		
	p	-	+	-	-		

+: Difference is significant

-: Difference is not significant

v: At constant volume (flasks stoppered)

p: At constant pressure (flasks not stoppered)

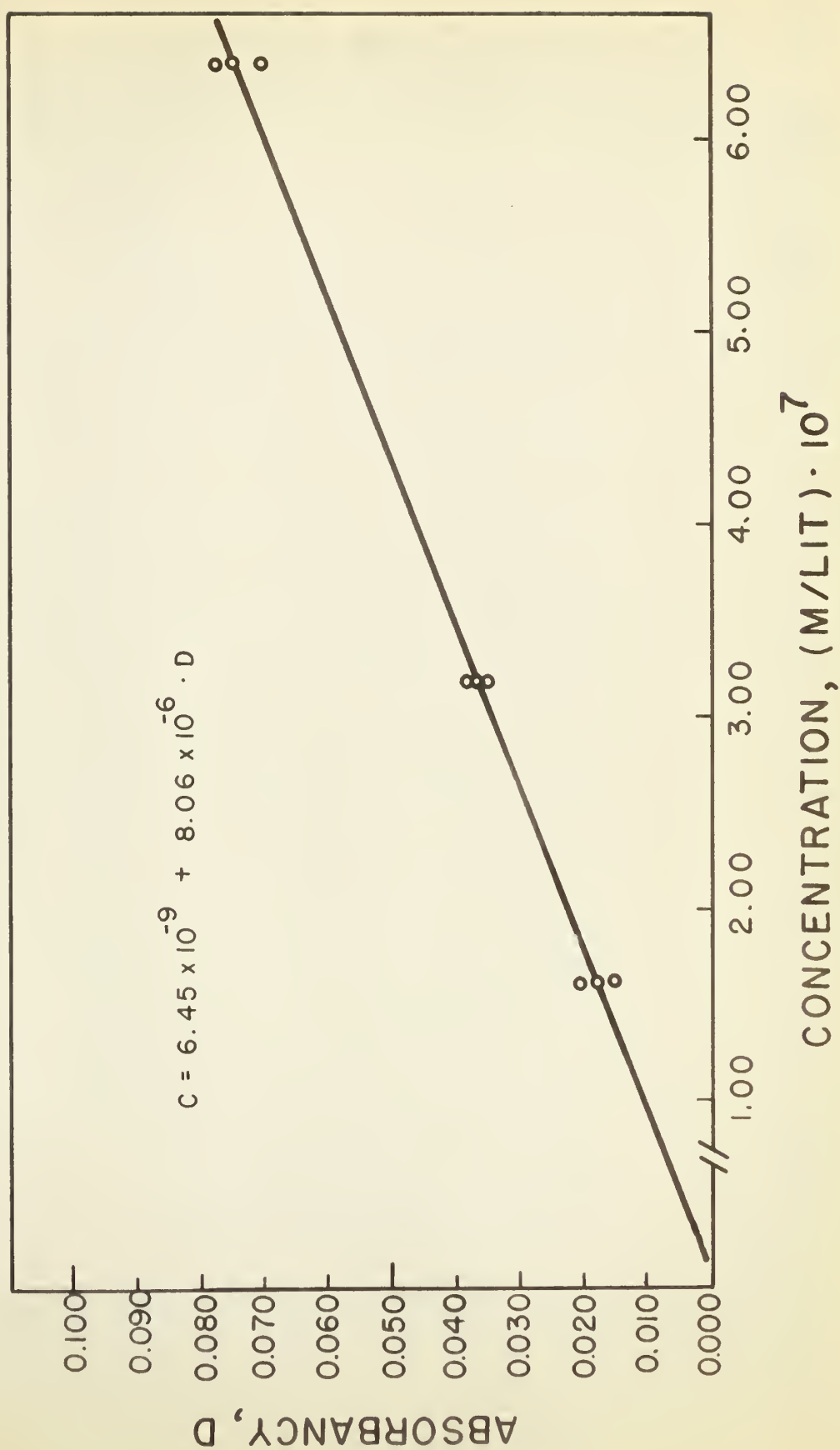


Fig. 2. Absorption curve of the 2-TBA-malondialdehyde pigment

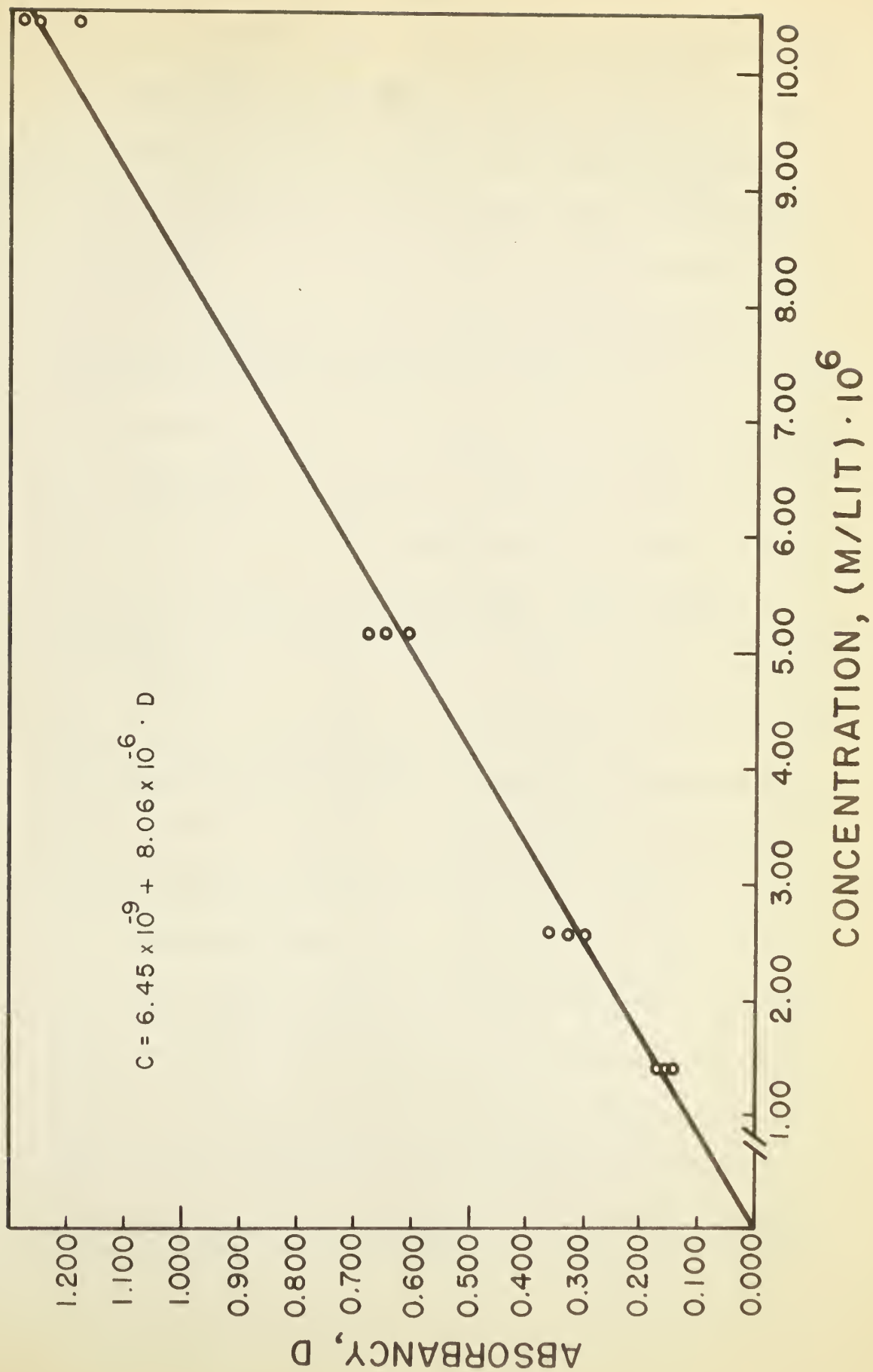


Fig. 3. Absorption curve of the 2-TBA-malondialdehyde pigment

mark with distilled water. The control was prepared by using the same volume of solution 8 as above, but instead of steam distillation the aliquot was directly transferred to a 100 ml volumetric flask and the volume adjusted with distilled water to about 50 ml. For the blanks 50 ml of distilled water was used. The absorbancy was measured as mentioned earlier. Altogether four different aliquots of solution 8 were used. The corresponding controls were run in duplicate while at each level ten condensates were collected. The pooled average recovery in per cent of control was found to be only 32.10 ± 0.02 , where 0.02 is the standard error of the mean. When the above mentioned experiment was repeated using a condenser temperature of 3°C , the average recovery was found to be $92.50 \pm 0.02\%$.

The absorbancy of the 2-TBA-2-unsaturated aldehyde pigment.

One hundredth of a mole of crotonaldehyde was weighed out and quantitatively transferred to a one liter volumetric flask. The volume was brought to the mark with a 30 % ($\frac{V}{V}$) solution of ethanol in distilled water to give stock solution I. A tenfold dilution of stock solution I yielded stock solution II. Working dilutions 1-10 were prepared as outlined in Table 5. From these solutions 5 ml aliquots were transferred to a 25 ml volumetric flask and 5 ml reagent of Täufel and Zimmermann

Table 5. Dilution of stock solution II. of crotonaldehyde
into working dilutions 1-10

Flask number	ml of stock II/100 ml
1	1
2	2
3	4
4	8
5	11
6	16
7	20
8	25
9	32
10	45

(88) was added. The reagent was prepared by dissolving 0.72 g 2-TBA in 90 ml glacial acetic acid on a steam bath. To this solution 2 ml of 0.01 M $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ was added and the volume made up to 100 ml. The flasks were stoppered and then heated in a 70°C water bath for 30 minutes. On cooling the volume was brought up to the mark with distilled water. The absorbancies were measured in a Beckman DU Spectrophotometer in 1 cm cells at 425 m μ and 0.2 mm slit width. The entire procedure was repeated once more. The results obtained are shown in Table 6. Every absorbancy entry is the average of four figures. The corresponding absorbancy versus concentration plot is shown in Fig. 4.

Comparison of the absorption spectra of 2-TBA with various carbonyls. Approximately 2.5×10^{-5} molar solutions of malondialdehyde, acetaldehyde, and acrolein were prepared in the aforementioned manner. Five ml aliquots were transferred to 25 ml volumetric flasks and to each 5 ml of 2-TBA reagent (saturated solution in glacial acetic acid, no Fe^{3+}) was added. After holding the solutions at room temperature for 12 hours the volumes were made up to the mark. A blank was prepared in the same way using 5 ml of distilled water in place of the carbonyl solutions. The visible absorption spectra of the three

Table 6. Absorption of the 2-TBA-crotonaldehyde pigment

Concentration, (M/ lit) $\times 10^6$	Absorbancy, D	D/(M/lit) $\times 10^{-4}$
2.05	0.008	0.390
4.10	0.016	0.390
8.20	0.034	0.415
16.40	0.086	0.524
22.55	0.144	0.639
32.80	0.380	1.159
41.00	0.960	2.341
51.25	1.880	3.668
65.60	2.140	3.262
92.25	2.130	2.309

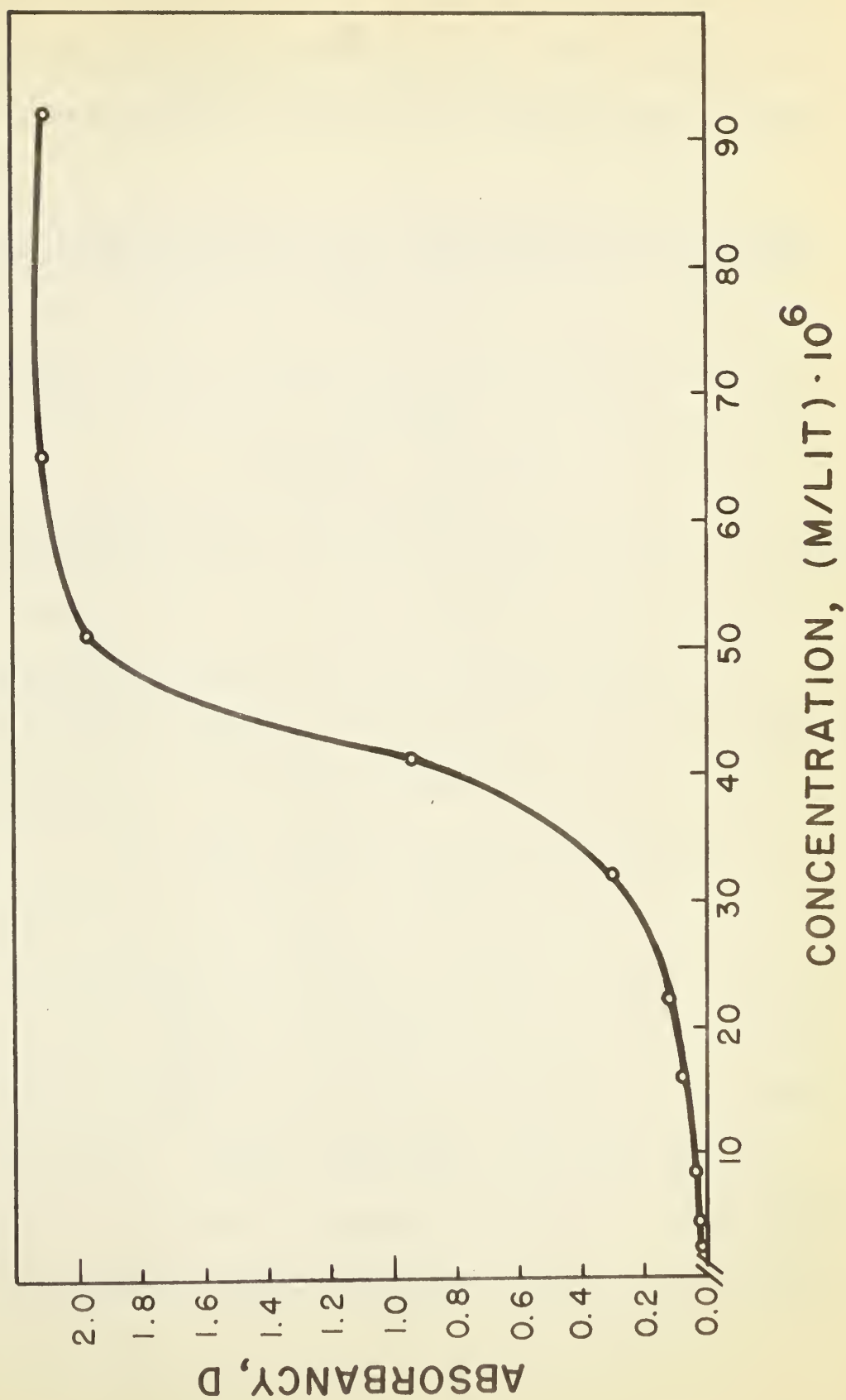


Fig. 4. Absorption curve of the 2-TBA-crotonaldehyde pigment

solutions against the common blank were obtained with a Cary Model 14 Recording Spectrophotometer. The wavelength and relative magnitude of the absorption peaks is shown in Table 7.

The molecular weight of the 2-TBA-malondialdehyde pigment.

From 0.125 moles of 2-TBA and 0.0625 moles of tetraethoxypropane 12.66 g of pigment was synthesized according to the procedure of Sinnhuber et al. (80). After drying the pigment in a vacuum oven ($P = 40$ mm Hg) at 60°C for four hours, it still contained about 8 % water by weight as checked by the Karl Fischer method. This water could be removed by drying the pigment in a thin layer over concentrated sulfuric acid in a desiccator for 48 hours. The pigment was found to be slightly hygroscopic; it slowly absorbed water from the atmosphere up to 6 %. The visible absorption spectrum of the pigment in a basic solution was found to be identical with that reported by Sinnhuber et al. (80). However, in acidic solution the peak of maximum absorption was shifted from 525 $\text{m}\mu$ to 532-535 $\text{m}\mu$, and the secondary peak at 425 $\text{m}\mu$ was replaced by one at 305 $\text{m}\mu$ (Table 7). Besides dilute alkali and pyridine (80), the pigment was found to be soluble in dimethyl sulfoxide, p-dioxane, and molten dl-camphor. This made possible the determination

Table 7. The wavelength of absorption peaks of three
2-TBA-carbonyl pigments

	Carbonyl		
	Malondi- aldehyde	Acetaldehyde	Acrolein
Wavelength of first peak, mμ	425	380	375
Wavelength of second peak, mμ	525	525	525
Ratio of absorbancies, $\frac{D}{D}$ (second peak) (first peak)	7.00	7.56	0.84

of the molecular weight by freezing point depression of dimethyl sulfoxide and camphor solutions according to the method of Daniels et al. (18) and by isothermal distillation from p-dioxane (61). The molar freezing point constant of dimethyl sulfoxide was found to be 4.05°C by the use of α -naphthylamine and p-phenylenediamine as standards. The elemental composition of the pigment was also obtained (61). The molecular weights by the different methods and the elemental composition are shown in Table 8 and Table 9 respectively. The most likely formula of the pigment seems to be $(\text{C}_{18}\text{H}_{12}\text{N}_6\text{S}_3\text{O}_6)_n$, where $n = 2, 3, 4, \dots$ depending on the medium.

The infrared spectrum of the 2-TBA-malondialdehyde pigment.

A sample of the solid pigment was dispersed in spectral grade potassium bromide. The mixture was fused into a clear, glassy disk in vacuum under 20 tons per square inch pressure. The infrared spectrum was recorded in a Perkin Elmer Model 421 Infrared Spectrophotometer. The position of the absorption peaks and the various bonds assigned to them (39) are shown in Table 10.

DISCUSSION

The results summarized in Table 4 indicate that the

Table 8. The molecular weight of the 2-TBA-malondi-
aldehyde pigment

Method	Molecular weight
Freezing point depression of dimethyl sulfoxide	1,650
Freezing point depression of camphor	1,020
Isothermal distillation in p-dioxane	2,280

Table 9. The elemental composition of the 2-TBA-malondialdehyde pigment

Element	Per cent of sample	Number of atoms , if S = 1
C	43.00	6
H	3.20	4-5
N	17.17	2
S	18.62	1
O ^a	18.77	2

(a) Was obtained by direct measurement and not by subtraction

Table 10. Absorption peaks of the 2-TBA-malondialdehyde pigment in the infrared spectrum

Peak, cm ⁻¹	Intensity of absorption	Bond	Group
3,425	W	N-H	Amide
2,900	W	C-H	=C=C-H
1,610	M	C=O, (C=C)	Amide
1,550	W	N-H	Amide
1,490	M		Skeletal vibration
1,350	S	C=S	
1,290	W	C-H	
1,200	W	C-OH	Enolization
1,160	W		Skeletal stretch
1,115	M		Skeletal stretch
1,000	W	C-H	=C=C-H
800	W	C-H	
770	W	C-H	
740	W	C-H	=C=C-H
670	W	C-S	C-SH
640	W	C-S	C-S-C

W= weak

M= medium

S= strong

reaction between 2-TBA and malondialdehyde can be brought to completion at room temperature in 4-12 hours. The blank under these conditions is of low non-specific absorption which allows an increase of sensitivity of malondialdehyde detection as compared with the reaction at constant volume and 70°C. However, in the case of routine analyses the time factor can prove to be prohibitive. Apart from the difference in sensitivity, the 4 to 12 hour condensation at room temperature is equivalent to a 30 minute - constant volume treatment at 70°C. The generally practiced condensation at 70°C for 30 minutes at constant pressure should be avoided because of the volatility of malondialdehyde.

The molar extinction coefficient of the 2-TBA-malondialdehyde pigment, 1.24×10^5 , is considerably lower than the value reported by Sinnhuber and Yu (79), 1.56×10^5 . The apparent cause of the deviation is the difference in pH, 1.79 versus 0.9 (79). This is to be expected according to the findings of Shepherd (76) who studied the nature of the 2-TBA-sulphadiazine pigment. The use of acetic acid in the Tüfelf and Zimmermann modification of the 2-TBA test (88) is desirable because it brings about a greater resistance of the amide linkages in 2-TBA against hydrolytic cleavage. The increased stability leads to the lowering of non-specific absorption (87). However, the corresponding

gain in sensitivity seems to be offset by the decrease in absorbancy.

The condenser temperature during the steam distillation of oxidized fats is usually 15-20°C. The poor recovery of malondialdehyde under these conditions, 32.10 %, shows that the condensate should be collected at 3°C or lower, when the recovery is raised to 92.50 %.

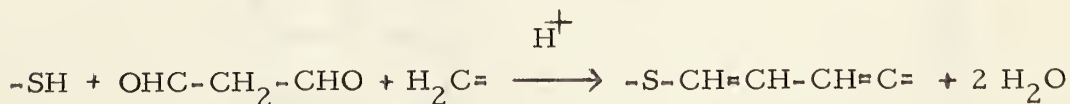
Kibrick et al. (40) reported that the sensitivity of the 2-TBA test could be increased by the presence of ferric ions. This increase is due to a reaction between carbonyls and 2-TBA which produces a pigment that does not follow Beer's Law (Fig. 4). Therefore, the addition of ferric ions should be restricted to cases where the amount of 2-unsaturated aldehydes is estimated (88), and then a calibration curve such as shown in Fig. 4 must be used. On the other hand, if the assigned role of the 2-TBA test is the estimation of lipid peroxides instead of carbonyls (40), then an explanation of the role of ferric ions is that they aid the oxidation of the sulfhydryl groups of two 2-TBA molecules into a disulfide bridge. The role of ferric ions in the estimation of 2-unsaturated carbonyls (88) can be assumed to be the same.

The successful condensation of three different types of carbonyls (Table 7) with 2-TBA at room temperature shows that

the generally accepted view of the role of malondialdehyde in oxidizing fats is in need of reconsideration, because both acetaldehyde and acrolein also have a definite absorption peak at 525 m μ . That the three pigments are not identical, however, is revealed by the different position of the secondary peaks, and by the difference of the absorbancy ratios (Table 7). There is probably only a small amount of malondialdehyde in oxidized fats, if any at all (79). On the other hand, various workers identified significant amounts of C₁-C₄ aldehydes besides higher ones in such widely different autoxidized products as milk fat (50), fish oils (99), and pork fat (27). Nevertheless, the conversion of these C₁-C₄ aldehydes into malondialdehyde according to the mechanisms outlined in the literature review must be assumed to be possible until proven otherwise. It is surprising that the only possible carbonyl of fat autoxidation that could react with 2-TBA in acidic solution and give rise to absorption at 520-540 m μ has been assumed to be malondialdehyde (67, 80), for Dox and Plaisance (20, 21) showed that under certain conditions 2-TBA will quantitatively precipitate furfural and condense with aromatic aldehydes. More recently Täufel and Zimmermann (89) condensed a large number of aldehydes with 2-TBA at 100°C. Some of the pigments showed an absorption maximum in the 496-535 m μ range. The relevance of this low

absorption range to the relationships shown in Table 7 is doubtful in the light of the work of Tarladgis et al. (87) because of the high condensing temperatures.

The simplest empirical formula for the 2-TBA malondialdehyde pigment which is in agreement with the results of elemental analysis is $C_6H_4N_2SO_2$. However, the evidence put forward by Sinnhuber et al. (80) and Tarladgis et al. (87) together with the bond requirements obtained by the interpretation of the infrared spectrum of the pigment (Table 10) demand an empirical formula of $\{C_{18}H_{12}N_6S_3O_6\}_n$. A corresponding structural formula is shown in Fig. 5. It is essentially the same as the product of a trimolecular condensation (two 2-TBA molecules and one malondialdehyde, shown in the literature review of this chapter), but the ratio of 2-TBA to malondialdehyde molecules is 3:2 instead of 2:1 (80). This means the condensation of malondialdehyde with the sulfhydryl group of 2-TBA as well as condensation at the number 5 carbon atom of 2-TBA. The reaction is as follows.



The formula weight of the compound is 504.54. The value of n that will satisfy the molecular weights listed in Table 8 must be 2, 3, or 4. The deviation of the molecular weights from small

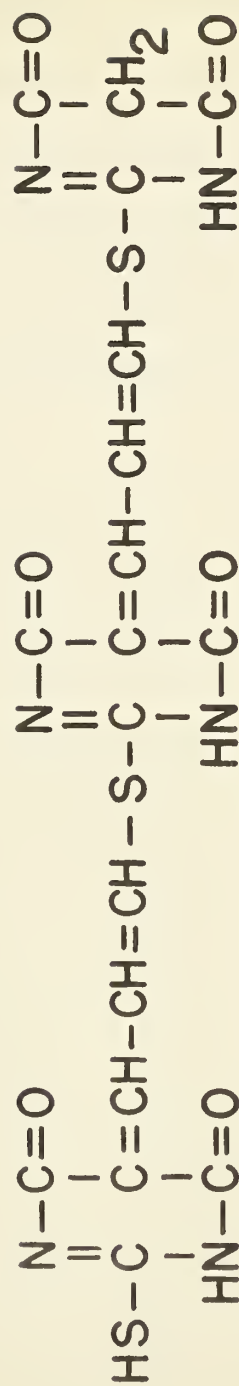


Fig. 5. Proposed structure of the 2 - TBA - malondialdehyde pigment

integer multiples of 504.54 is no doubt due partly to experimental errors. However, the presence of various co-polymer fragments in solution must also be assumed. It was noticed that the 2-TBA-malondialdehyde pigment crystals dissolved with a characteristic purple colour in alkaline water, pyridine, and dimethyl sulfoxide, but the colour in p-dioxane was light brown. This could be caused by molecular rearrangement, but more likely by a difference in the degree of polymerization.

LIPOXIDASE CATALYZED OXIDATION OF EMULSIFIED
LINOLEIC AND LINOLENIC ACID IN THE PRESENCE
OF A COMPETITIVE INHIBITOR

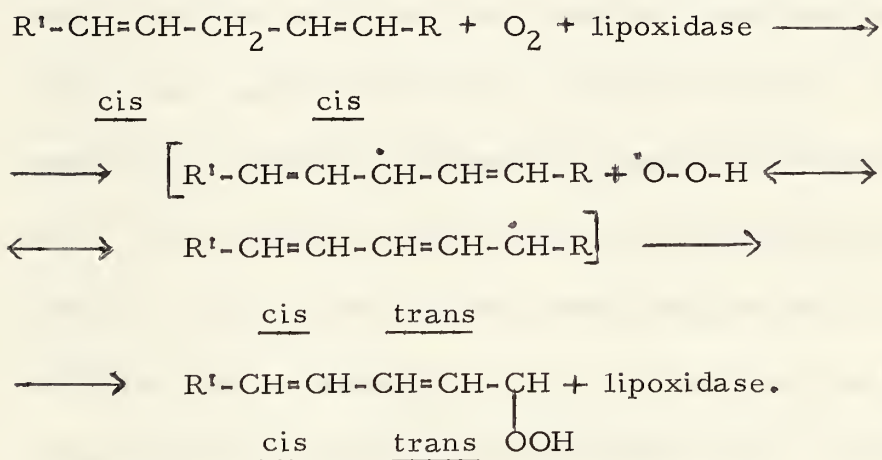
INTRODUCTION

Certain legumes and cereal grains contain lipoxidase which is a coenzyme free, fat oxidizing enzyme. The most important sources of lipoxidase are soybeans, wheat, barley, peanuts, and flax seeds. The presence of this enzyme in animal tissues has never been demonstrated.

Under customary conditions of processing and storing, lipoxidase can cause the oxidative deterioration of fats, and thus the flavour, in many foodstuffs. Such susceptible products can be unblanched frozen peas, corn, and beans or crackers that were made from lipoxidase bleached flour. Moreover, some vegetable oils that were obtained by carbon tetrachloride or trichlorethylene extraction of ground seeds or by the water-and - pressure displacement method usually have an inferior keeping quality.

Lipoxidase is specific for the cis, cis 1,4-pentadiene groups of the appropriate polyunsaturated fatty acids. Thus, the cis-9, cis-12 isomer of linoleic acid, the cis-9, cis-12, cis-15 isomer

of linolenic acid, and the cis-5, cis-8, cis-11, cis-14 isomer of arachidonic acid, their alkali salts, and alkyl esters are subject to lipoxidase catalyzed oxidation. The rates of oxidation of these compounds per one 1,4-pentadiene group are equal under identical conditions (77). The trans-trans polyunsaturated fatty acids and the monounsaturated fatty acids are competitive inhibitors of lipoxidase catalysis (19). The reaction products are optically active, cis-trans, conjugated, monomeric hydroperoxides (71). The most likely reaction mechanism of lipoxidase action is as follows (85).



Object. One of the least clarified problems of fat autoxidation is the nature of free radical initiation. It has been established from both kinetic and thermodynamic points of view (16) that the most likely sources of free radicals are peroxides. Theoretically therefore, the question concerning the origin of the

first hydroperoxide molecule in an autoxidizing system is a very important one. Tappel et al. (86) have shown that the energy of activation of the lipoxidase catalyzed oxidation of sodium linoleate is 4.3 Kcal/mole at pH 9 and in the presence of a large concentration of the substrate. Under comparable conditions the energy of activation of the autocatalytic linoleate oxidation is nearly four times greater (84), and the energy of activation of ordinary chemical reactions is around 40 Kcal/mole. It is safe to assume then that in any system that contains polyunsaturated fatty acids and lipoxidase at a suitable temperature, and this may be the case with the above mentioned food-stuffs, the chances of lipoxidase catalyzed oxidation, and especially a lipoxidase initiated free radical autoxidation are good. The existence of an induction period during autoxidation, however, points to the fact that some large number of free radicals must be present before the active phase can begin. Because the all cis polyunsaturated fatty acids are usually accompanied by the aforementioned competitive inhibitors, the question arises whether under the customary conditions of processing and storage the production of hydroperoxides by lipoxidase catalyzed fat oxidation is thermodynamically more probable than by direct oxygen uptake. In other words, the problem is whether hydroperoxides that are produced by

lipoxidase can be a significant part of the free radical pool of the induction period. The following experiment was conducted in order to answer this question through the estimation of the activation energies involved.

MATERIALS AND METHODS

Samples of linoleic and linolenic acids were obtained from the Hormel Foundation. Two grams of linolenic acid and four grams of linoleic acid, both composed of 50 mole per cent of pure cis isomer and 50 mole per cent of trans isomers, were dissolved in absolute ethanol to 25 ml volumes to yield 0.2872 moles/l and 0.5704 moles/l solutions respectively. There were no peroxides present, because one ml aliquots of these substrates diluted with 20 parts of air free, 0.1 N H_2SO_4 yielded no titratable amounts of iodine.

Crystalline lipoxidase was obtained from the Nutritional Biochemicals Corporation. A quantity of 0.1 g, approximately 9.76×10^{-7} moles, was dissolved in a 10^{-3} molar phosphate buffer and made to 1 liter. A 5-ml aliquot of this stock solution was diluted to 1 l with the same buffer to give the required concentration of enzyme.

A Sargent Oil Stability Apparatus was used with the

following modification. A polyethylene coil for cooling the thermostat liquid below room temperature was wound around the test tube rack. A 5°C coolant from a refrigeration unit was circulated through the coil at a constant rate. It is important to note that the apparatus is equipped with a preheating manifold that brings the temperature of the air to the set value of the thermostatic bath before it is forced through the samples. The air was scrubbed by a 0.02 M phosphate buffer 0.001 M in EDTA solution in order to remove traces of copper and iron, and then by distilled water. The air was dried by passage through a tower filled with anhydrous CaCl_2 .

The temperature of the oil stability apparatus was set to one of the following values: 15, 20, 25, or $30 \pm 0.05^\circ\text{C}$. Twenty-ml aliquots of the deaerated, buffered enzyme, $[\text{E}]^1$, solution were introduced into 25 x 200 mm test tubes, and after thermal equilibration, 1-ml aliquots of the substrate, $[\text{S}]^1$, solution were introduced, and the test tubes were fitted with the aeration tubes. The airflow was started and kept at 0.5 l/min. and 25 mm Hg pressure per 12 samples as measured at 25°C at the air intake manifold.

Using the reported turnover number for lipoxidase with emulsified substrates (53), the substrate to enzyme ratio, $[\text{S}]^1 / [\text{E}]^1$, was adjusted to give not more than 10 % substrate change

in the first 25 minutes at 30^o C. This required an air flow rate between 0.2 and 0.9 l/min. It was observed that a flow rate outside of this range destroyed the fatty acid in alcohol emulsion through coalescence.

Five fatty acid samples and a blank were tested at each temperature. The pH of the linoleic and linolenic acid emulsions was determined both before and after the aeration by a Metrohm Precision Compensator E 322. The glass and saturated calomel electrode system was standardized with 0.05 molar phthalate solution, pH 4.01 at 25^o C; the setting checked out against a 0.01 molar borax solution, pH 9.18 at 25^o C. Therefore, the linearity of the electrode response was accepted for the 4.01 < pH < 9.18 range at 25^o C.

After 20 ± 0.17 minutes from the time of the start of the airflow, the test tubes were transferred to a boiling water bath for five minutes to terminate the reaction, and kept at 4^o C until the estimation of hydroperoxides took place. The determination was carried out according to the modified Wheeler iodometric method (Chapter VI).

RESULTS AND DISCUSSION

Hydrogen ion concentration. Table 11 shows the pH values of

Table 11. pH of the substrate emulsions of linoleic and
linolenic acid

Temperature of the reaction °C	pH at 25°C			
	Before reaction		After reaction	
	Lino- leic	Lino- lenic	Lino- leic	Lino- lenic
15	6.75	6.80	6.70	6.80
20	6.75	6.80	6.72	6.77
25	6.75	6.80	6.71	6.74
30	6.75	6.80	6.67	6.74

the substrate emulsions. These values are very close to the reported optimum pH value of 6.5 for methyl linoleate and methyl linolenate with soybean lipoxidase [81]. Variation within triplicate samples was found to be as much as between temperatures; thus, the changes according to temperature are not significant.

Rate constants and activation energies. Since in this experiment $[S] > [E]$, it can be assumed that the reaction is of zero order for not more than 10 per cent change of the initial substrate concentration. That is, $d[O_2]/dt = K$. Thus, the rate constant, K , is calculated from the following equation.

$$K = \frac{V \times (10^3/20.9) \times 10^{-3} \times 0.005}{20} = 1.23 \times 10^{-5} \times V \text{ moles per liter per minute}$$

where:

V = ml of 0.01 N $Na_2S_2O_3$ solution used by the liberated iodine

20.9 = volume in ml of 1 ml alcoholic substrate and 20 ml enzyme solution

0.005 = millimoles of oxygen per substrate site, equivalent to 1 ml 0.01 N $Na_2S_2O_3$ solution

20 = time of reaction in minutes

The activation energies were calculated from the integrated

Arrhenius equation.

$$\log K = \frac{-E_a}{2.303 \times R} \times (1/T) + C$$

where:

E_a = energy of activation, cal/mole

T = temperature of the reaction in absolute degrees

R = universal gas constant

C = a constant

The slope of the $(1/T) \times 10^3$ versus $\log (K \times 10^5)$ line was calculated by the method of least squares. This relationship was preferred instead of the reverse one, because it was found that the variation in temperature during the reaction is less than the variation in rate between samples. The individual rate constants were used for the calculation, because basing the computation on the averages only would have meant loss in degrees of freedom, and thus loss in precision of estimation. The calculations are set out in Tables 12 and 13. The activation energies are listed in Table 14.

Variation of the reaction rate with 10°C temperature change;

the Q_{10} values. The nearly perfect linearity of the \log

$(K \times 10^5)$ versus $(1/T) \times 10^3$ plot, Fig. 6, shows that the E_a

values for linoleic and linolenic acids do not change with temper-

Table 12. Calculation of the activation energy and Q_{10} value of linolenic acid oxidation

Sample	Temp. °C	$10^3/T$	V(ml)	V-Bk(ml)	$K \times 10^5$	$\log (K \times 10^5)$	$(1/T) \times 10^3$ $\log (K \times 10^5)$
Bk	15	3.47	.10				
1			4.15	4.05	4.97	.6964	2.42
2			4.10	4.00	4.91	.6911	2.40
3			4.05	3.95	4.84	.6848	2.38
4			4.10	4.00	4.91	.6911	2.40
5			4.15	<u>4.05</u>	<u>4.97</u>	.6964	2.42
				4.01	4.92		
Bk	20	3.41	.10				
1			5.50	5.40	6.62	.8209	2.80
2			5.65	5.55	6.81	.8332	2.84
3			5.60	5.50	6.74	.8287	2.82
4			5.50	5.40	6.62	.8209	2.80
5			5.50	<u>5.40</u>	<u>6.62</u>	.8209	2.80
				5.45	6.68		
Bk	25	3.35	.10				
1			7.40	7.30	8.96	.9523	3.19
2			7.50	7.40	9.08	.9581	3.21
3			7.45	7.35	9.02	.9552	3.20
4			7.35	7.25	8.89	.9489	3.18
5			7.60	<u>7.50</u>	<u>9.20</u>	.9638	3.23
				7.36	9.03		
Bk	30	3.30	.20				
1			10.05	9.85	12.08	1.0821	3.57
2			9.95	9.75	11.96	1.0777	3.56
3			10.10	9.90	12.14	1.0842	3.58
4			10.05	9.85	12.08	1.0821	3.57
5			10.10	9.90	12.14	1.0842	3.58
		67.65		9.85	12.08	17.7730	59.95

Continuation of Table 12

$$\left[-E_a = \frac{2.303 \times R}{-\text{slope}} \right]$$

$$\begin{array}{l} \text{Slope} = \frac{59.95 - (1,202.34/20)}{16.22 - (315.88/20)} = \frac{-0.167}{0.426} = -0.392 \\ (1/T) \text{ on } \log K \end{array}$$

$$E_a = \frac{4.576}{0.392} \times 10^3 = 11,673$$

$$Q_{10} = \frac{(12.08/4.92)}{1.5} = 1.64$$

Table 13. Calculation of the activation energy and

Q₁₀ value of linoleic acid oxidation

Sample	Temp °C	$10^3/T$	$V(\text{ml})$	$V\text{-Bk}(\text{ml})$	$K \times 10^5$	$\log (K \times 10^5)$	$(1/T) 10^3 \times 10^5$ $\log (K \times 10^5)$
Bk	15	3.47	.10				
1			3.45	3.35	4.11	.6138	2.13
2			3.35	3.25	3.99	.6010	2.08
3			3.55	3.45	4.23	.6263	2.17
4			3.40	3.30	4.05	.6075	2.11
5			3.45	<u>3.35</u>	<u>4.11</u>	.6138	2.13
				3.34	4.10		
Bk	20	3.41	.10				
1			4.65	4.55	5.58	.7466	2.54
2			4.90	4.80	5.89	.7701	2.63
3			4.85	4.75	5.83	.7657	2.61
4			4.70	4.60	5.64	.7513	2.56
5			4.75	<u>4.65</u>	<u>5.70</u>	.7559	2.58
				4.67	5.73		
Bk	25	3.35	.20				
1			6.85	6.65	8.16	.9117	3.05
2			6.80	6.60	8.10	.9085	3.04
3			6.80	6.60	8.10	.9085	3.04
4			6.90	6.70	8.22	.9149	3.06
5			6.70	<u>6.50</u>	<u>7.97</u>	.9015	3.02
				6.61	8.11		
Bk	30	3.30	.20				
1			9.65	9.45	11.59	1.0641	3.51
2			9.70	9.50	11.65	1.0663	3.52
3			9.65	9.45	11.59	1.0641	3.51
4			9.65	9.45	11.59	1.0641	3.51
5			9.60	9.40	11.53	1.0618	3.50
		67.65		9.45	11.59	16.7175	56.30

Continuation of Table 13

$$\begin{array}{l} \text{Slope} \\ (1/T) \text{ on } \log K \end{array} = \frac{56.30 - (1,130.94/20)}{14.54 - (279.47/20)} = \frac{-.247}{.566} = -0.436$$

$$E_a = \frac{4.576}{.436} \times 10^3 = 10,495$$

$$Q_{10} = \frac{11.59/4.10}{1.5} = 1.88$$

Table 14. Activation energies and Q_{10} values of
lipoxidase catalyzed oxidation of linoleic
and linolenic acid

Substrate	E_a (cal/mole)	Q_{10}
Linoleic acid	1.05×10^4	1.88
Linolenic acid	1.17×10^4	1.64

1. $\int_0^1 (x^2 + 1) dx = \left[\frac{x^3}{3} + x \right]_0^1 = \frac{1}{3} + 1 = \frac{4}{3}$

2. $\int_0^1 (x^2 + 1) dx = \frac{4}{3}$

3. $\int_0^1 (x^2 + 1) dx = \frac{4}{3}$

x	$(x^2 + 1) dx$	$\int_0^1 (x^2 + 1) dx$
0	1	1
1	2	2

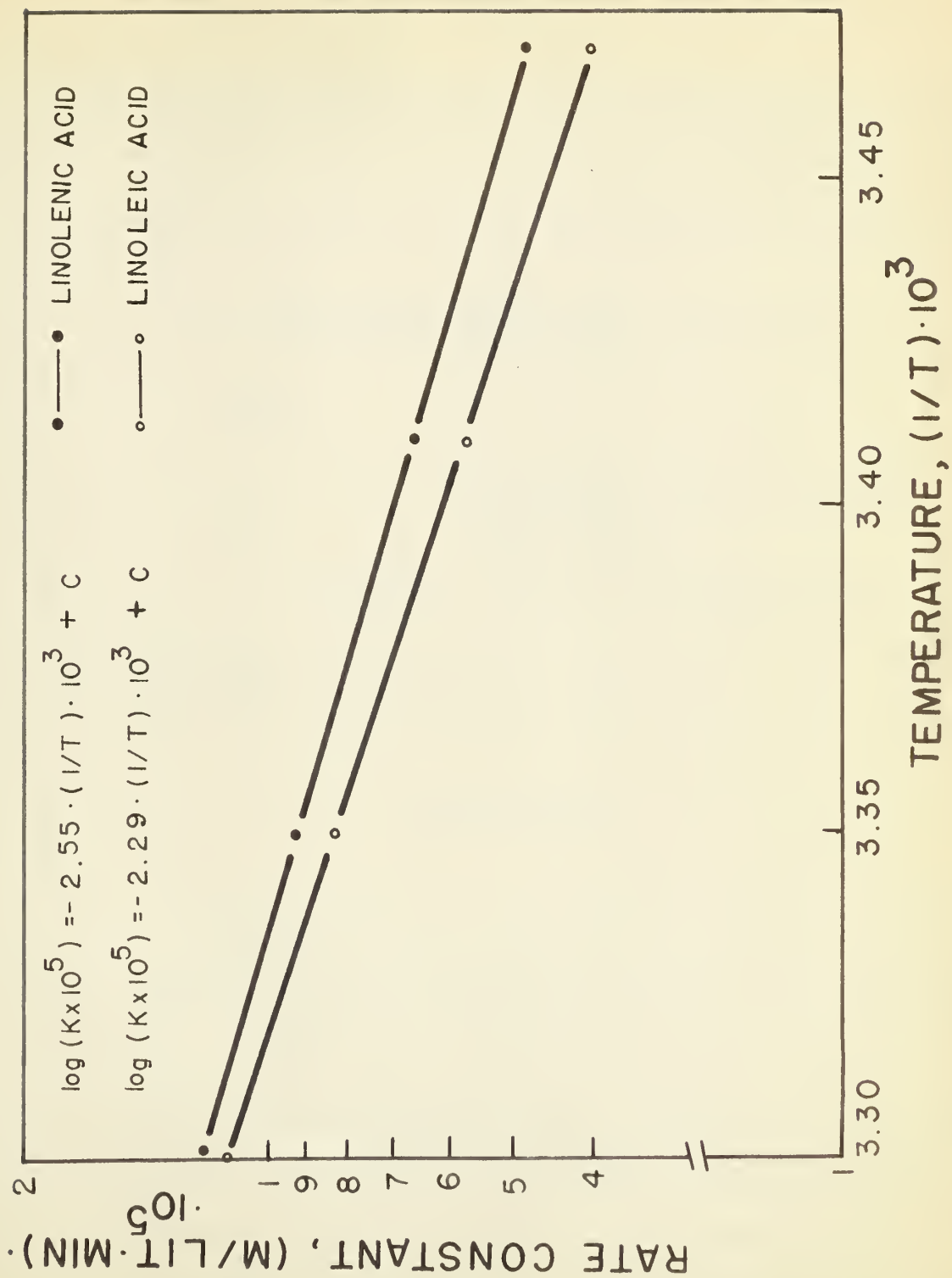


Fig. 6. Arrhenius equations; Lipoxidase system

ature in the 15-30°C range. Therefore, the Q_{10} values can be calculated for the entire range and are given in Table 14.

The turnover numbers, T.N. Defined as the number of moles of substrate altered per sample per second divided by the number of moles of enzyme per sample that brought this change about.

$$\text{T.N.} = \frac{\sqrt{(0.005 \times 10^{-3}) / (20 \times 60)}}{0.1 / (200 \times 50 \times 102,400)} = 43.7 \times \bar{V}$$

where:

\bar{V} = the average number of ml of 0.01 N $\text{Na}_2\text{S}_2\text{O}_3$ solution used for the iodine liberated

102,400 = molecular weight of lipoxidase

The obtained values are given in Table 15.

CONCLUSIONS

Although there are some restrictions concerning the applicability of these findings to the oxidative deterioration of foods, the difficulties are mainly in the realm of differences that exist between simplified model systems and natural ones. However, this experiment was not designed to elucidate the nature of such differences, but merely to decide upon the thermodynamic probability of a process. Since all hydrocarbon

Table 15. Turnover numbers of lipoxidase catalyzed
oxidation of linoleic and linolenic acid

Temp. °C	Turnover Number	
	Linoleic acid	Linolenic acid
15	145	175
20	205	240
25	290	320
30	410	430

per-oxidations result in a decrease of the free energy of the system, it is mainly the magnitude of the activation energy that has the greatest influence on the rate of such reactions.

The somewhat higher activation energy of linolenic acid per 1,4-pentadiene group, Table 14, is probably caused by the steric inhibition brought about by one oxidized 1,4-pentadiene group on the adsorption of the intact one on the enzyme surface. This effect seems to be greater than lowering of the activation energy through the increased resonance stabilization of linolenic acid as compared to linoleic acid.

When evaluating the rate constants and activation energies, it should be remembered that there is no way of measuring the amount of autocatalytically generated hydroperoxides in the presence of hydroperoxides formed enzymatically. Blanks obviously do not help in this case because they lack the amount of free radical initiation that is available in the lipoxidase system. During the early stages of oxidation it is very probable that enzyme molecules surpass the action of free radicals due to their larger number.

According to Holman (34) the optimum temperature for lipoxidase catalyzed hydroperoxide production is 30°C , and the Q_{10} value is 1.6 for the -1.5 to $+18.5^{\circ}\text{C}$ range. With the acid substrates used there seemed to be no inactivation of enzyme

up to 30°C, and the Q_{10} values are comparable with those of Holman (34).

The turnover numbers, Table 15, are valid only for a lipoxidase system where 50 % of the substrate is a competitive inhibitor, and their magnitude confirms only the catalytic nature of lipoxidase action.

It can be concluded, with some reservations, that from a thermodynamic point of view the lipoxidase catalyzed oxidation of polyunsaturated fatty acids in the presence of equal amounts of competitive inhibitors is feasible as a catalytic reaction. The average activation energies are still only approximately one fourth of those of ordinary chemical reactions. The generation of a hydroperoxide molecule by lipoxidase, on the average, is nearly as possible as the generation of one during the active phase of autoxidation of pure polyunsaturated fatty acids. Even in the presence of equal concentrations of competitive inhibitors, lipoxidase can generate hydroperoxides. Thus, during the customary processing and storing of fatty foodstuffs lipoxidase can contribute to the shortening of the induction period.

THE EFFECT OF DIFFERENT PARTIAL PRESSURE OF
OXYGEN, CONSTANT VOLUME AND TEMPERATURE ON
THE ULTRAVIOLET LIGHT CATALYZED OXIDATION
OF A BUTTER CONCENTRATE

INTRODUCTION

The most pronounced effect of catalysts on the course of fat oxidation is the shortening of the induction period (13). During this time interval the rate controlling factor of oxidation is the speed with which the primary free radicals are generated. Ultraviolet light accelerates the formation of free radicals which results in a decrease of the time necessary for the onset of the active phase of fat oxidation. The shortening of the induction period, however, is by no means the only effect of ultraviolet light on oxidizing fats. It has been shown (52) that the simultaneous catalytic oxidation of the secondary products of autoxidation, especially aldehydes, can also occur. As far as the flavour stability of fats is concerned, the formation of free radicals and peroxides on one hand, and the removal of carbonyls on the other are two opposing processes. Unfortunately, the two processes are of unequal rate, and the rate of the oxidation of already

formed carbonyls must be slower than the rate of free radical and peroxide formation as otherwise no fat would develop oxidative rancidity. It would be of considerable significance to know if the rates of the above processes are capable of alteration in a way that would result in the formation of smaller amounts of carbonyls. Among the many factors that might affect these rates, the effect of the partial pressure of the oxygen, constant volume and temperature were investigated.

EXPERIMENTAL

The samples were obtained from 12 oz. cans (The Butter Marketing Board, Brisbane, Australia). The cans had been stored mostly at 4^o C. The age of the product was over six years at the time of the experiment. Some pertinent data concerning the composition and quality of the product are given in Table 16. The cans were cooled to -15^o C before opening and were kept at that temperature in a nitrogen atmosphere until the sampling could take place.

A batch of samples to be tested was made up from triplicate 5.00 g portions of each can. Four batches, two batches per can, constituted a lot that underwent simultaneous irradiation. The 5.00 g portions were weighed into Astell Roll Tube Bottles

Table 16. Concentration of some of the constituents
in Australian Butter Concentrate

	Can	
	I.	II.
Age (years)	> 6	>6
Flavour	Acceptable	Acceptable
Butterfat ¹ (% , wt)	90	90
Hydrogenated vegetable oil ¹ (% , wt)	3	3
Sodium chloride ^a	2.6	2.7
Water ^b	.015	.011
Copper ^c (p.p.m.)	<.01	<.01
Iron ^c (p.p.m.)	.54	.58

(1) Manufacturer's specifications

(a) Amperometric, AgNO₃

(b) Karl Fischer Method

(c) Polarographic (Chapter II)

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(Astell Laboratory Service Co. Ltd., London, England). After ensuring that no trace of fat adhered to the inside surface of the bottle neck, they were fitted with clean, #3200 Vacutainer stoppers (Becton, Dickinson and Co., Columbus, Nebraska). Preliminary investigation showed that this arrangement provided a completely airtight seal. The bottles were placed in a thermostatic bath, at 25°C and the pressure in the bottles was reduced to 40 mm Hg by the means of a #24 hypodermic needle, a rotary vacuum pump, and a McLeod gauge. Using a gasometer, 13.1 ml/ 25°C of oxygen gas was introduced into a third of the samples. Another third received the same amount of nitrogen, and the rest was left evacuated. (The 13.1 ml/ 25°C of gas and the residual air have a total pressure of approximately 760 mm Hg at 60°C in a Roll Tube Bottle of 20 ml volume containing 5.00 g of butter.) The tubes were laid in closest packing but in one plane on a synchronous rotator (60 r.p.m.) designed for this purpose. The rotator was mounted in an oven kept at $60 \pm 2^{\circ}\text{C}$ with the tops of the tubes three inches from an ultraviolet lamp (Fisher 11-984; dominant wavelength 2537 Å; radiation intensity 5×10^{-4} watts per cm^2 per second). Two lots were irradiated and two were not. After 12 hours the samples were cooled to 25°C and the pressure of the residual gas was measured as above. The change in

pressure, ΔP , was converted into mEq/kg oxygen absorbed by the following expression.

$$\text{mEq/kgO}_2 = 4 \times 10^5 \frac{\Delta P \times V}{RT}$$

The samples were subsequently cooled to -15°C and held at that temperature until analysis.

The following tests were run in duplicate.

- a) Index of refraction, A.O.C.S. Official
Method Cc7-25 (59)
- b) Dienoic and trienoic acid content by
alkali isomerization with tertiary
butoxide (97)
- c) Peroxide values according to the modified
Wheeler procedure (Chapter VI)
- d) Saturated and unsaturated carbonyls by the
2,4-dinitrophenylhydrazine method (6).

The results obtained are shown in Table 17. Every entry is the average of 48 measurements. On the other hand, Table 18 shows the change in the samples with respect to the controls. Rows 4,6,8 of Table 18 show the respective changes expressed in terms of per cent of total oxygen uptake in a particular column. The summations of these values along columns are contained in row 9. The following expression serves to convert

Table 17. Oxidation of Australian Butter Concentrate

Tests	Control	Not irradiated			Irradiated		
		O ₂	N ₂	Vacuum	O ₂	N ₂	Vacuum
Oxygen uptake, mEq/kg	1 -	8.50	5.10	2.40	9.20	5.00	3.60
Peroxide value, mEq/kg	2 0.00	0.10	0.05	0.05	0.00	0.00	0.00
Index of refraction, n _D ⁵⁰	3 1.4511	1.4510	1.4510	1.4511	1.4512	1.4508	1.4512
Dienoic acid, % {wt}	4 2.34	1.65	1.72	1.85	2.17	2.46	1.99
Trienoic acid, % {wt}	5 1.43	1.16	1.05	1.11	1.39	1.40	1.27
Saturated carbonyls, mM/kg	6 3.43	3.68	3.72	3.85	5.88	5.20	4.88
Unsaturated carbonyls, mM/kg	7 0.46	0.30	0.15	0.26	0.00	0.00	0.00

Table 18. Oxidative changes in Australian Butter Concentrate

Tests		Not irradiated				Irradiated			
		O ₂	N ₂	Vacuum		O ₂	N ₂		Vacuum
		a	b	c		a	b	c	
Dienoic acid; control-sample, %(wt)	1	0.69	0.62	0.49		0.17	-0.12		0.35
	2	0.27	0.38	0.32		0.04	0.03		0.16
Trienoic acid; control-sample, %(wt)	3	0.10	0.05	0.05		0.00	0.00		0.00
	4	1.18	0.98	2.08		0.00	0.00		0.00
Saturated carbonyls	5	0.25	0.29	0.42		2.45	1.77		1.45
	6	5.88	11.37	35.00		53.26	70.80		80.56
Unsaturated carbonyls	7	-0.16	-0.31	-0.20		-0.46	-0.46		-0.46
	8	-3.76	-12.16	-16.67		-10.00	-18.40		-25.56
Per cent of O ₂ uptake accounted for		3.30	0.19	20.41		43.26	52.40		55.00

millimoles of carbonyl per kg into milliequivalents of oxygen per kg.

$$\Delta \text{ mEq/kg}_{\text{oxygen}} = k \cdot \Delta \text{ mM/kg}_{\text{carbonyl}}$$

where:

k = 2,4,6 for 1,2,3 oxygen atoms
per carbonyl molecule respectively

All of the heated or heated and irradiated samples developed oxidized flavours. The flavour stability of the type of butter used in this experiment and that of similar products has been evaluated by Hansen et al. (28) in an earlier study.

DISCUSSION

The reason for selecting the Australian Butter Concentrate instead of freshly churned butter is twofold. First of all, the product has a remarkable keeping quality (28) which has never been demonstrated with unmodified commercial butter. The figures of Table 17 show that the control has significant amounts of the two types of carbonyls. Nevertheless, the flavour of the product betrayed no signs of oxidative deterioration. This means that under certain conditions oxidative changes can take place with apparent absence of off flavours. Therefore, it was

decided to test the relative importance of composition versus environment with the aim to gain some insight into the possibility of improving keeping quality by the means of environment alone. The second reason for selecting the Butter Concentrate was the age of the product. Because of the long, anaerobic storage, all of the oxidative changes could be assumed to be in equilibrium. Accordingly, it was expected that small effects of temperature, oxygen, and ultraviolet light that would not alter the composition of a fresh product to a measurable extent, because of the induction period, could give rise to a series of definite changes in a relatively short period of time.

The accuracy of the refractive index measurements is ± 2 units in the fourth decimal place. Consequently, there is no significant difference between the control and the samples (Table 17, 3rd row). The sum of mole refractions of the samples remains constant. In contrast to this, there is a definite change in the uptake of highly polar oxygen (1st row of Table 17). The suitability of obtaining the degree of oxygen uptake by the measurement of the isothermal pressure drop would be questionable on two grounds: the formation of gases and the formation of volatile components of significant vapor pressure at 25°C. There is no evidence available on gas formation of any kind during autoxidation

apart from that of carbon dioxide. It is shown in Chapter VI that the amount of carbon dioxide produced under even more severe conditions is exceedingly small. The question of vapours is not so simple. There is indirect evidence concerning their presence to be discussed later, and to that extent the estimation of oxygen uptake might be in error.

According to Schaffer et al. (74) milk fat at 70°C will develop oxidative rancidity in an atmosphere that contains more than 0.80 per cent oxygen by volume. Assuming that the composition of the dissolved gas in the fat is the same as above it, they show that the above figure corresponds to 0.009 ml_(STP) O₂/g fat. This is equivalent to 1.6 mEq O₂/kg fat, but it was found that both irradiated and not irradiated samples developed oxidative off flavours after considerably larger oxygen uptake (rows 4, 6 of Table 18 versus row 1 of Table 17). The effect of partial pressure of oxygen on oxidative stability cannot be definitely stated until the nature of oxygen solubility and diffusion in fats is clarified. The same point is well illustrated by Bolland's work (9) who showed by oxidizing ethyl linoleate, that at very low partial pressure of oxygen the rate of oxygen uptake is directly proportional to the partial pressure. At the same time, at pressures of over 200 mm Hg the rate is wholly independent of the partial pressure.

The increase in peroxide value and carbonyls account only for a fraction of the oxygen uptake (rows 4,6,8,9 of Table 18). The low peroxide values are caused by the instability of peroxides at the applied temperature. This phenomenon was observed first by Holm (33). It can be seen on comparison of row 3 with rows 5 and 7 of Table 18 that considerable oxidative changes can take place at very low peroxide levels. Accordingly, the value of the widespread use of the estimation of the peroxides present as a measure of the state of oxidative deterioration of fats, or as the predetermined end point in stability tests is questionable. The determination of carbonyls and the evaluation of the flavour are no doubt more useful methods.

The figures shown in row 6 of Table 18 merit the following comment. It would be expected that lowering of the partial pressure of oxygen will retard the formation of carbonyls. In fact, just the opposite happens so far as saturated carbonyls are concerned, while the formation of unsaturated carbonyls (row 8) is retarded. According to the Mass Action Law, the formation of saturated carbonyls must be accompanied by the liberation of some gaseous entity that is in separate equilibrium with oxygen, if the effect of reduced oxygen partial pressure is to be explained. If this product exerts a significant pressure at 25⁰C, then the described measurement of oxygen uptake must be erroneous to

an unknown extent.

The role of ultraviolet light as a catalyst of fat oxidation has been demonstrated {13}; In the present experiment the question is whether the radiation can penetrate the Astell Bottles. The differences between the two major columns of Table 18 show that it can. The wavelength and intensity of the transmitted radiation, however, cannot be ascertained. Irradiation favours the preservation of dienoic and trienoic acids {rows 1,2 of Table 18} probably by aiding the conjugation of the double bonds which results in compounds that are more resistant to oxidative cleavage. On the other hand, it aids the decomposition of peroxides and unsaturated carbonyls {rows 3,7 of Table 18}. Oxidation of the sample in dark leads to a greater loss of dienoic and trienoic acids. At the same time, there is a slight increase in peroxides and a lowering of unsaturated carbonyl decomposition. These comparisons indicate that the difference between heat and heat plus light is quantitative rather than qualitative.

As 2-enals, and 2-dienals are the carbonyls mostly responsible for the oxidized flavours of fats, a moderate heat treatment of an oxidized fat in the presence of ultraviolet light and reduced pressure should improve the flavour. This would be a process similar in effect to that used in the high temperature deodorization of fats and oils {1}.

THE ACCELERATED OXIDATION OF MILK FAT, COTTON- SEED OIL, AND LARD

INTRODUCTION

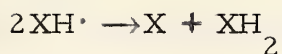
For the time being, the use of antioxidant is the best possible measure for the counteraction of oxidative deterioration of edible fats. Nevertheless, it must be emphasized that antioxidants do not prevent the occurrence of oxidative processes but merely slow them down. The most important gain is in the time during which the product remains relatively free from objectionable changes. If a suitable antioxidant is at hand, the satisfactory transit of the fat or fat containing food from production to consumption can be assured. The experience of food processors and distributors, however, has shown during the past fifty years that such an antioxidant is not always available. It is common knowledge now that a universal antioxidant does not exist, and that for practically every substrate and for every set of conditions a new antioxidant is required. Little is known about the mechanism of antioxidation or the relationship between structure and antioxidant efficiency. Therefore, the selection of a suitable antioxidant is still done on a trial and error basis. The most direct way of checking such a compound is done under

practical processing and storing conditions with organoleptic tests. Unfortunately, this method takes too much time, and organoleptic tests are highly subjective, showing not only individual but regional variations as well. Large scale manufacturing and distribution of foods would require less subjective results in advance and frequently on short notice.

Antioxidants are classified as primary antioxidants and synergists. The former are polyphenols, and the latter are compounds such as ascorbic, citric, and phosphoric acids. They are used in the 0.001 - 0.500 per cent range. A comparison of various antioxidants and synergists on weight rather than mole per cent basis is more convenient for the industry. The effect of polyphenols is enhanced by alkyl substitution in the ortho or para positions (90). Synergists are thought to act through the deactivation of prooxidant metal contaminants by chelation (23). Since no data are available for the stability constant or the solubilities of the complexes in fats or in water-in-fat emulsions, the exact mechanism involved is not known. The most likely mode of action of an antioxidant, XH_2 , is through hydrogen donation.



The semiquinone formed would undergo a dismutation reaction (12).



The quinones, X, have never been isolated from autoxidizing fats that contain polyphenols. In spite of the notable argument put forward by Uri (93), there is no substantial evidence to support the view that antioxidants react only with the $\text{RO}_2\cdot$ type free radicals but not with the $\text{R}\cdot$ ones. The RH type molecules are more stable than the corresponding hydroperoxides. The problem is especially intriguing if one recalls the well known fact that fats having a high level of hydroperoxides cannot be stabilized by any amount of antioxidant, although hydroperoxides are thought to be the precursors of the $\text{RO}_2\cdot$ type free radicals (3).

The distinction between primary antioxidants and synergists is arbitrary. Ascorbic acid is a chelating agent, but it can be a prooxidant in milk by reducing copper(II) and iron(III) ions to the more effective copper(I) and iron(II) states. On the other hand, it will be shown that ascorbyl palmitate is a very effective antioxidant for milk fat in the presence of significant amounts of copper and iron.

The effectiveness of an antioxidant is usually evaluated through its effect on the lengthening of the induction period of an autoxidizing fat. In the Active Oxygen Method (59) the test

is carried out at 97.7°C by bubbling through the samples clean, dry air at a controlled rate until an arbitrarily chosen peroxide value is reached. The time required to attain this value as compared to the control samples is the measure of the anti-oxidant efficiency. Still another method is the measurement of the inhibition of the production of some compound formed during the accelerated test.

The contradictory results published on the effectiveness of certain antioxidants under supposedly identical conditions demonstrate that the above mentioned criteria are unsatisfactory. Apparently small differences in the concentration of initial free radicals, metals, water, acidity, and fatty acid composition of the fat will cause disproportionately great variation in the induction period or the accumulation of peroxides. It was shown in Chapter V that the peroxide values, depending on temperature and ultraviolet radiation, have no bearing on the accumulation of carbonyls or other secondary products. In the present experiment the effectiveness of antioxidants will be checked by several different tests to decide if there is a more suitable measure of antioxidant effectiveness than the induction period versus peroxide value relationship.

The literature contains no information on the effect of different fatty acid composition on the course of autoxidation.

If such differences are important at all, they should manifest themselves through qualitative and quantitative changes in the products of autoxidation. Accordingly, three fats of significantly different fatty acid composition were selected for the accelerated oxidation tests. Special attention was paid to the demonstration of such qualitative and quantitative differences among the oxidation products of cottonseed oil, milk fat, and lard that might explain the low taste threshold value of carbonyls in milk fat.

Ascorbyl palmitate has been selected as one of two anti-oxidants because of its surface active properties (47). From a theoretical point of view such antioxidants should provide more protection against oxidation of most natural fats, containing water in fat emulsions, than either primary antioxidants or synergists, because they can align themselves preferentially at the fat-water droplet interface where the chance of oxidative attack is the greatest. On the other hand, cetyl gallate has been selected because of its potentially greater effectiveness, through better solubility in fats, than that of its widely used homolog: propyl gallate (91). A further aim of this experiment will be to correlate and possibly improve the various tests for autoxidized fats.

The results of antioxidant evaluation at high temperatures naturally will not be applicable without further considerations to

low temperature processing and storing conditions. At the same time, the accelerated oxidation tests on cottonseed oil and lard will have definite bearing on such problems as the stability of shortenings during deep frying processes.

EXPERIMENTAL

Milk Fat

Preparation of samples. Four pounds of butter were purified by washing with hot water and dried at 60°C and 20 mm Hg pressure in a rotating evaporator. This operation was carried out in a nitrogen atmosphere. The clear, dry milk fat was stored in the dark at -17°C in a nitrogen flushed, airtight jar until further use. The copper and iron contents were determined according to the method described in Chapter II (Table 19). The approximate fatty acid composition of milk fat can be obtained by referring to Table 20 compiled from the collected data of Kirschenbauer (41). One lot of samples contained no antioxidant, the other three contained 0.01 % cetyl gallate, 0.01 % ascorbyl palmitate, or 0.02 % ascorbyl palmitate by weight respectively. Measurements proved cetyl gallate to be completely ineffective as a milk fat antioxidant at 100°C . However, this fact is shown only in connection with the development of free fatty acids (Fig. 7), and it is to be under-

Table 19. Concentration of copper and iron in three
edible fats, determined by polarography

Metal	Cottonseed oil	Milk fat	Lard
Copper, p.p.m.	0.14	0.02	≤0.01
Iron, p.p.m.	1.80	0.95	0.62

Year	Area	Population	Notes
1950	100	100	Initial population
1960	100	100	Initial population

Table 20. Fatty acid content in weight % of three edible
fats after Kirschenbauer

Systematic name of acid	Cotton-seed oil	Milk fat	Pig fat
Butanoic		2.6-4	
Hexanoic		1.4-2.0	
Octanoic		0.5-1.5	
Decanoic		1.6-2.7	
Dodecanoic		1.7-3.7	
Tetradecanoic	0.5-3.0	8.0-13.0	0.7-1.3
Hexadecanoic	17-23	25-32	25-31
Octadecanoic	1-3	8-13	11.0-16.5
Eicosanoic	0.1-1.5	0.4-2.4	
9-Decenoic		0.1-0.3	
9-Dodecenoic		0.1-0.4	
9-Tetradecenoic		0.6-1.6	0.0-0.3
9-Hexadecenoic	0.8-2.5	1.6-5.0	2-5
9-Octadecenoic	18-44	26.5-34	40-51
9,12-Octadecadienoic	34-55	2.5-4	3-12
C ₂₀ -C ₂₂ - enoic		0.3-1.8	1.7-3.0

stood when interpreting the results shown in Table 21. Twelve samples of each lot were subjected to accelerated oxidation (83) at $100.0 \pm 0.05^{\circ}\text{C}$ for 24 hours in a Sargent Oil Stability Apparatus. The apparatus was modified as follows: Air, from a compressed air cylinder was led through a purification train before entering the manifold system. The first of four connected hydrometer cylinders, each of 50 mm O.D. and 375 mm height was filled to 200 mm height with a solution 0.001 M in EDTA and 0.02 M in phosphate buffer in order to remove traces of cupric and ferric ions. The common logarithms of the stability constants of the metal-EDTA complexes in this medium are approximately 19 for copper(II) and 26 for iron (III) ions (8), sufficiently large to retain both ions. The second cylinder contained a saturated solution of barium hydroxide, and the third one distilled, deionized water to the same height. The fourth one a 150 mm column of anhydrous calcium chloride between two layers of glass wool. The air flow was obtained by simultaneous measurement of the temperature, pressure and flowrate of the air between the reduction valve and the purification train. It was adjusted to 0.75 liter per minute at 21°C and 738.6 mm Hg pressure per 12 samples. The exit gases from each sample were led through a solution of 10 ml 2-thiobarbituric acid reagent for the determination of saturated and unsaturated carbonyls

(Chapter III) or 10 ml of distilled water for pH measurements.

The 2-TBA solutions were renewed in the traps at 8 hour intervals. At the same time samples were withdrawn for polarographic peroxide determination. After oxidation the samples were stored under identical conditions with the control until the analysis could be conducted. The entire procedure was repeated twice. The oxidized samples and unoxidized controls were subjected to the following tests.

Estimation of dienoic and trienoic acid content. Conducted according to the method of White and Quackenbush (97) by alkali isomerization with tertiary butoxide. During the first trial the control levels were found much too low. However, when the sample size was increased to about five-fold of the suggested (97) amount, 0.1 g, the results were more in keeping with those reported in reference texts (37). The average figures that were obtained are shown in rows 2 and 3 of Table 21.

Index of refraction. The A.O.C.S. Official Method Cc7-25 (59) was used. Because the determination was checked primarily as a potential tool for detecting incipient autoxidation, the measurements were restricted to samples without antioxidant. The results are shown in row 4 of Table 21.

Table 21. The effect of ascorbyl palmitate on the oxidation of milk fat at 100°C for 24 hours

Tests			Control	Ascorbyl palmitate, %		
				0.00	0.01	0.02
			1	2	3	4
Weight of oxidized sample, g		1		29.5	29.3	29.5
Polyunsaturated fatty acid, %	dienoic	2	2.120	0.147	0.111	0.083
	trienoic	3	0.182	0.071	0.036	0.027
Index of refraction, $n_{D}^{50^{\circ}\text{C}}$		4	1.4511	1.4518		
pH of fat extract		5	6.86	5.00	6.10	6.37
Volatile water soluble fatty acids liberated in 24 hours, pH of 25 ml solutions		6	5.7	2.7	3.1	3.6
Peroxide value, mEq/kg	iodo-metric	7	13.4	278.2	156.6	55.4
	polarographic	8	0.00	458	350	164
Carbonyls, mM/kg	saturated	9	9.9	102.4	35.2	12.4
	unsaturated	10	0.7	49.6	29.6	10.0
2-TBA numbers, mg/kg	malondialdehyde	11	0.2	32.8	20.8	9.6
	croton aldehyde	12	8.9	235.2	249.4	211.8

Determination of free fatty acids. Originally the A.O.C.S.

Official Method Ca 5a-40 (59) was used. Later, however, the 95 % ethyl alcohol as solvent was replaced by the more convenient 1:1 ether-ethanol mixture. Parallel determinations using the two solvents yielded results that were not significantly different at the 1 % level using Student's t-test. The results obtained are shown in Fig. 7.

pH of aqueous fat extracts and exit gas trap solutions. The water soluble constituents from one g fat samples were extracted with 25 ml water at 50°C by vigorous shaking for five minutes. The mixtures were held at 25°C for one hour, and cooled to 4°C.

When the fat phase solidified, a portion of the aqueous layer was withdrawn into 7 ml polyethylene cups. The pH was measured at 25°C as described in Chapter IV. The results are shown in row 5 of Table 21. The volume of the exit gas trap solutions was made up to 25 ml and the pH determined. The values are given in row 6 of Table 21.

Iodometric peroxide values. Wheeler's method (96) was found to be satisfactory with the following modifications. The solvent was deaerated before dissolving the fat sample by bubbling purified nitrogen through, and the titration was completed in a nitrogen

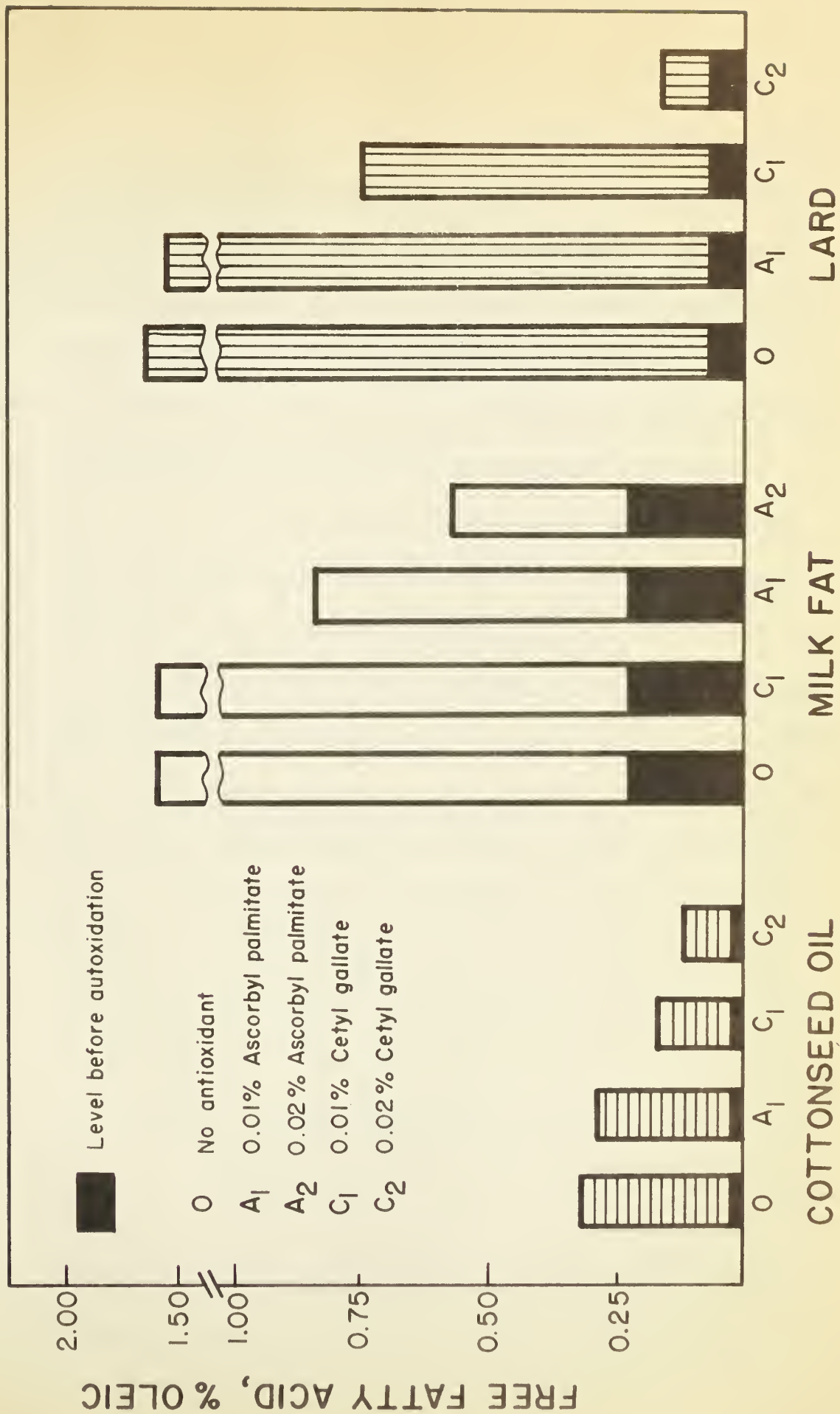


Fig. 7. Effect of antioxidants on autoxidizing fats at 100°C

atmosphere. Moreover, prior to the addition of potassium iodide one drop of normal ammonium molybdate solution was added to the reaction mixture (14). The reasons for these modifications are as follows. Wheeler observed that peroxides of heated fats oxidize iodide much slower than those from unheated ones (96). Therefore, the one minute reaction time prescribed in his determination had to be increased for the complete reduction of peroxides. However, this carried with it the danger of oxygen interference from the atmosphere and the possibility of iodine absorption by the unsaturated systems present. The use of a nitrogen atmosphere has been dealt with by Lea (46), and Heaton and Uri (29). On the other hand, the role of metals in the reaction mixture has apparently been misinterpreted. In the presence of atmospheric oxygen, oxidation of iodide is greatly increased by metal ions leading to high results (29); but as soon as oxygen is excluded the presence of metal ions is desirable because they catalyze the slow reaction between iodide and cyclic and polymer peroxides that are present in fats oxidized at high temperature. The possibility of almost instantaneous titration of iodine in the presence of metal ions under anaerobic conditions is a definite asset. The ease with which metal ions or metal complexes, especially molybdate (14), decompose peroxides has been confirmed by several workers (2, 57). Thus, the use of metal

catalysts during anaerobic peroxide determinations is highly desirable.

The use of "Soluble Starch," marketed by several companies, as an end point indicator was found to give negative errors in the order of 4-9 % as compared with whole starch solutions. The bluish-red colouration of "Soluble Starch" has shown that the product is a high molecular weight dextrin and not true starch. The colouration was less discernible than that produced by iodine alone. Ordinary corn starch was used for satisfactory end point detection.

The results of iodometric peroxide determinations of milk fat are shown in row 7 of Table 21.

Polarographic peroxide values. The polarograms were recorded with a Sargent Model XV Polarograph. A 1:1 mixture of benzene (thiophene free) and methanol (spectral grade) was used as solvent with 0.3 molar lithium chloride as supporting electrolyte (49). In the presence of 0.01 % ethyl cellulose (44) the resolution of the waves could be improved. The use of mangan(II) ions, 10^{-5} moles per 0.06-0.10 g fat, as an internal standard made it possible to relate the diffusion currents due to peroxides: i_p , and mangan(II) ions: i_{Mn} to peroxide values by the following equation (69, 36).

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$$P.V. = 14.70 \frac{i_P}{i_{Mn}} \cdot \frac{1}{m}, \text{ where}$$

m is the weight of the fat sample in grams. The open circuit drop time was set to approximately 3.8 seconds, the flow rate of mercury at 2 mg per second, and the temperature at 25° C. The calculated peroxide values are shown in row 8 of Table 21 and in Fig. 8.

Determination of carbonyls by the 2,4-dinitrophenyl-hydrazine method. The use of 2,4-dinitrophenylhydrazine for the colorimetric estimation of carbonyls in 10^{-6} - 10^{-3} molar solutions originated from Lappin and Clark (45). The 2,4-dinitrophenylhydrazones are obtained in a polar solvent such as water, alcohol, acetic acid, or ethyl acetate under the catalytic influence of hydrochloric acid. The actual pigment, of quinoidal structure, is derived from the phenylhydrazones by a rearrangement in a strongly alkaline medium. Beer's Law was found to apply in the above concentration range. The wavelength of maximum absorption is 480 mμ, and the molar extinction coefficient is 2.72×10^4 . Both values are independent of the structure of aliphatic aldehydes, ketones, and certain diketones. However, the solvents mentioned are unsuitable for fats. The introduction of benzene (70) has led to three major problems. Carbonyls tend

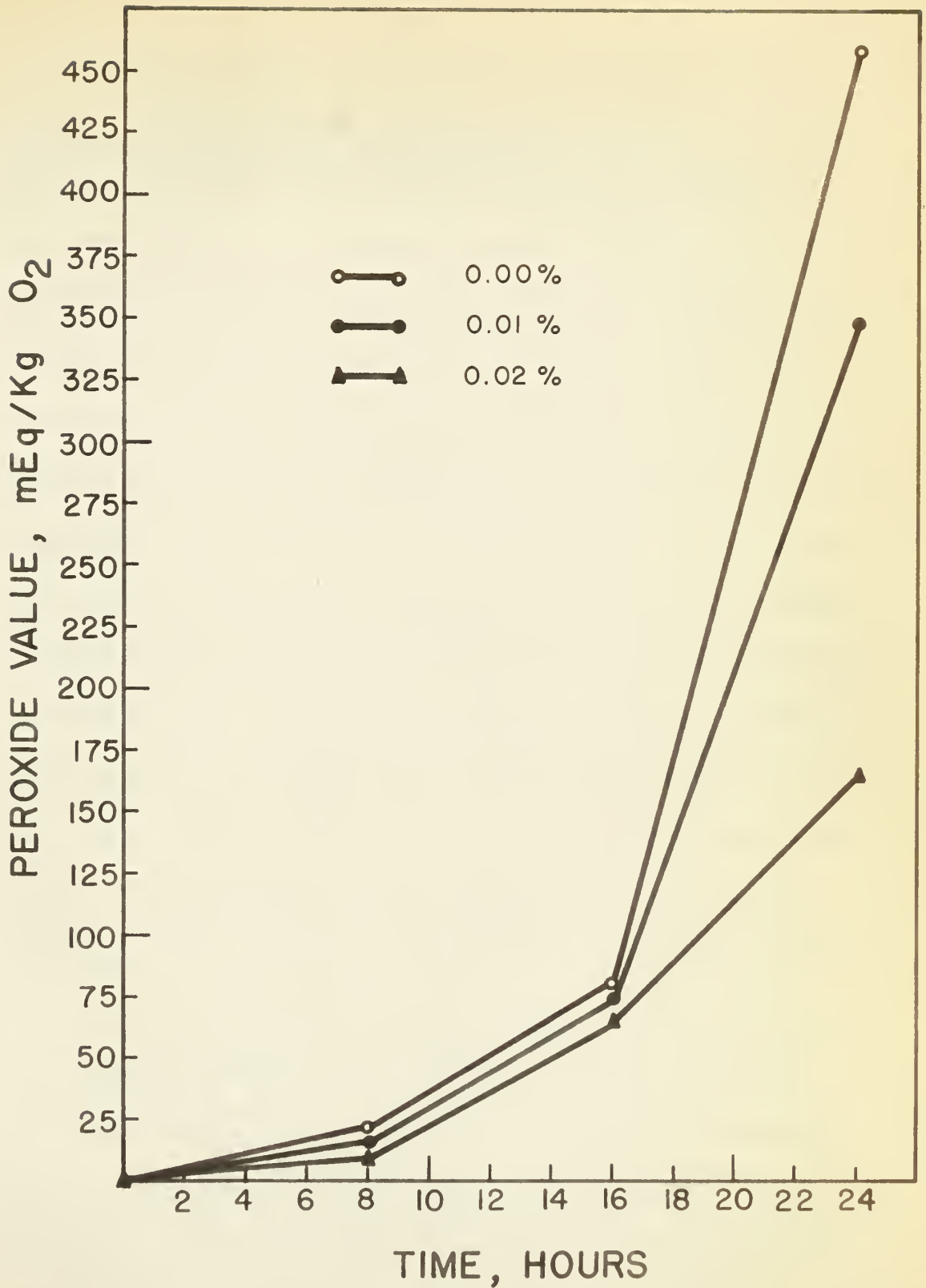


Fig. 8. The effect of ascorbyl palmitate levels on the development of polarographic peroxides of milk fat at 100° C

to decompose in benzene solutions under the influence of diffuse daylight. Alkali chloride precipitates out during the rearrangement of phenylhydrazones, and there is a rapid fading of the developed colour. Only the second problem has been solved satisfactorily. The use of trichloroacetic acid as catalyst was decided upon by Henick et al. (31) the potassium salt of which is soluble in benzene. The same workers derived two formulas, based on the absorption of hexanal and crotonaldehyde 2,4-dinitrophenylhydrazones, for the calculation of the amount of saturated and unsaturated carbonyls. The absorbancy readings were taken at 430 and 460 m μ exactly 10 minutes after the addition of the base. Colours developed under identical conditions in benzene withdrawn at five minute intervals for thirty minutes had the same absorbancies. Such measurements repeated in the same environment but under different lighting conditions and with different fats gave identical results. It can be concluded that during the first thirty minutes after the dissolution of fat samples in benzene there is no measurable carbonyl decomposition. In other respects the test was carried out as described by Berry and McKerrigan (6). The absorbancy readings were taken with a Beckman DU Spectrophotometer. The saturated and unsaturated carbonyl contents of milk fat samples are shown in rows 9 and 10 respectively of Table 21.

2-TBA numbers. The determination of steam volatile saturated and unsaturated carbonyls was conducted according to the method of Täufel and Zimmermann (88) with the modifications described in Chapter III. The absorbancies were converted to moles per liter concentrations by the use of Fig. 4 in the case of unsaturated carbonyls. The corresponding concentrations of crotonaldehyde in mg per kg fat were calculated from the following equation.

$$\text{mg/kg} = 10^3 \frac{V \times d \times 70.09}{m} \cdot C$$

where:

V = volume of the final solution in milliliters

d = dilution; total volume of distillate/volume of aliquot taken into the final solution.
Both in milliliters.

m = weight of fat sample in grams

70.09 = Mwt of crotonaldehyde

C = Moles per liter concentrations (Fig. 4)

The concentrations of malondialdehyde were calculated from the following relation.

$$\text{mg/kg} = 10^3 \frac{72.065}{1.24 \times 10^5} \times \frac{V \times d}{m} D_{530 \text{ m}\mu}$$

where:

72.065 = Mwt of malondialdehyde

1.24×10^5 = Molar extinction coefficient of the
2-TBA-malondialdehyde pigment

$D_{530 \text{ m}\mu}$ = The absorbancy of the final solution

V, d, m = As above

The results obtained are shown in rows 11, 12 of Table 21, and in Figures 9, 10.

Polarography of autoxidized milk fat. From fat polarograms (49, 44) of autoxidized milk fat, cottonseed oil, and lard samples the half-wave potentials were obtained graphically. The number of electrons transferred per atom of reducible substance, \underline{n} , was calculated in the same manner as in Chapter II. The average results obtained with the standard error of the mean, are shown in column 2 of Table 22.

In order to clarify the identity of the polarographic waves of autoxidized fats under the above conditions (49, 44), the following high purity compounds were polarographed alone at a concentration level of approximately 0.4 millimoles per liter: crotonaldehyde, butyraldehyde, heptaldehyde, 2-pentanone, 2-heptanone, hydrogen peroxide, and mangan(II) chloride. Only those compounds are shown in Tables 23 and 24 which gave diffusion currents. To check on possible overlaps the latter compounds were also polarographed together. The diffusion current constants and half-wave potentials

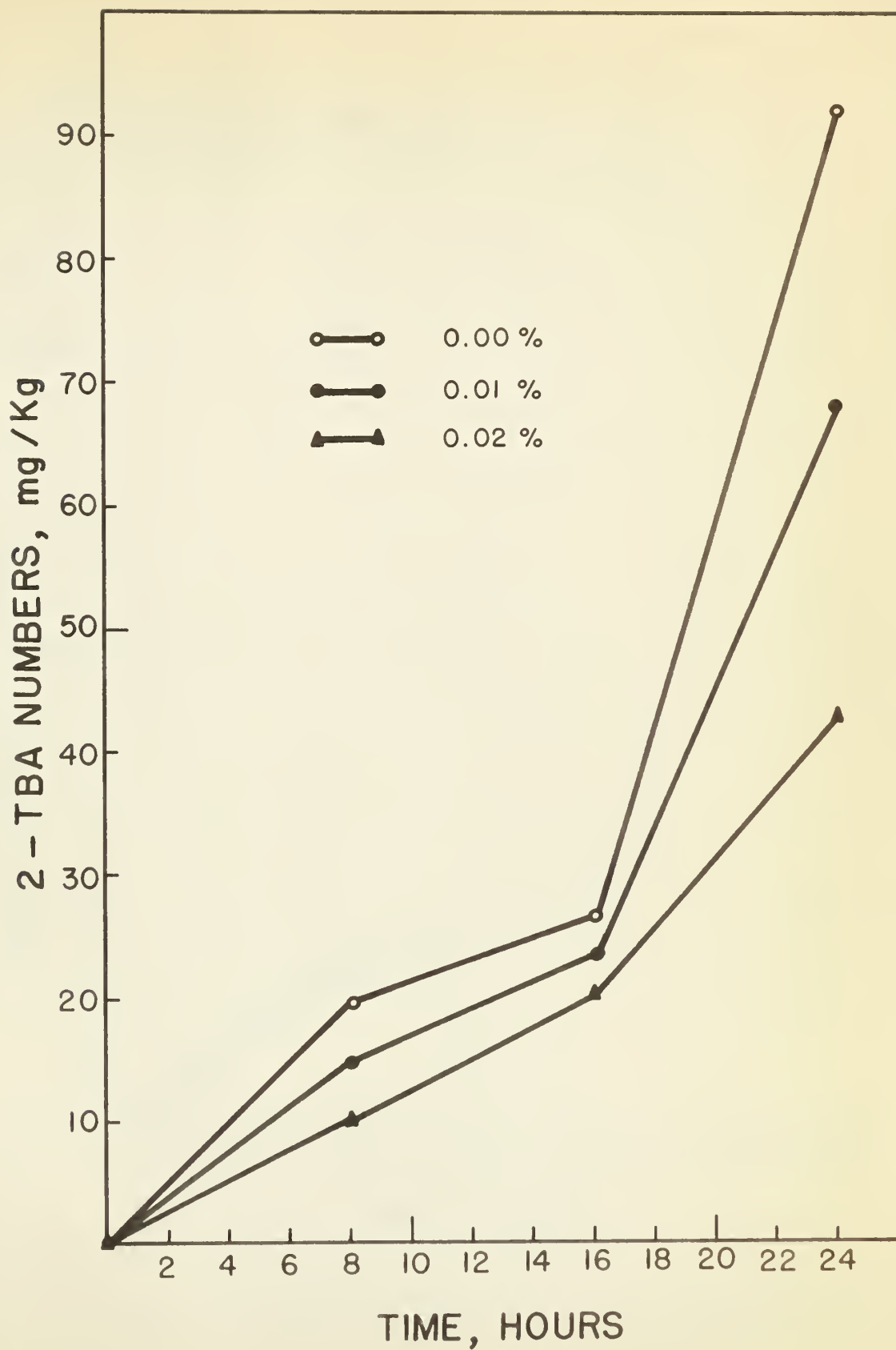


Fig. 9. The effect of ascorbyl palmitate levels on the evolution of malondialdehyde from milk fat at 100°C

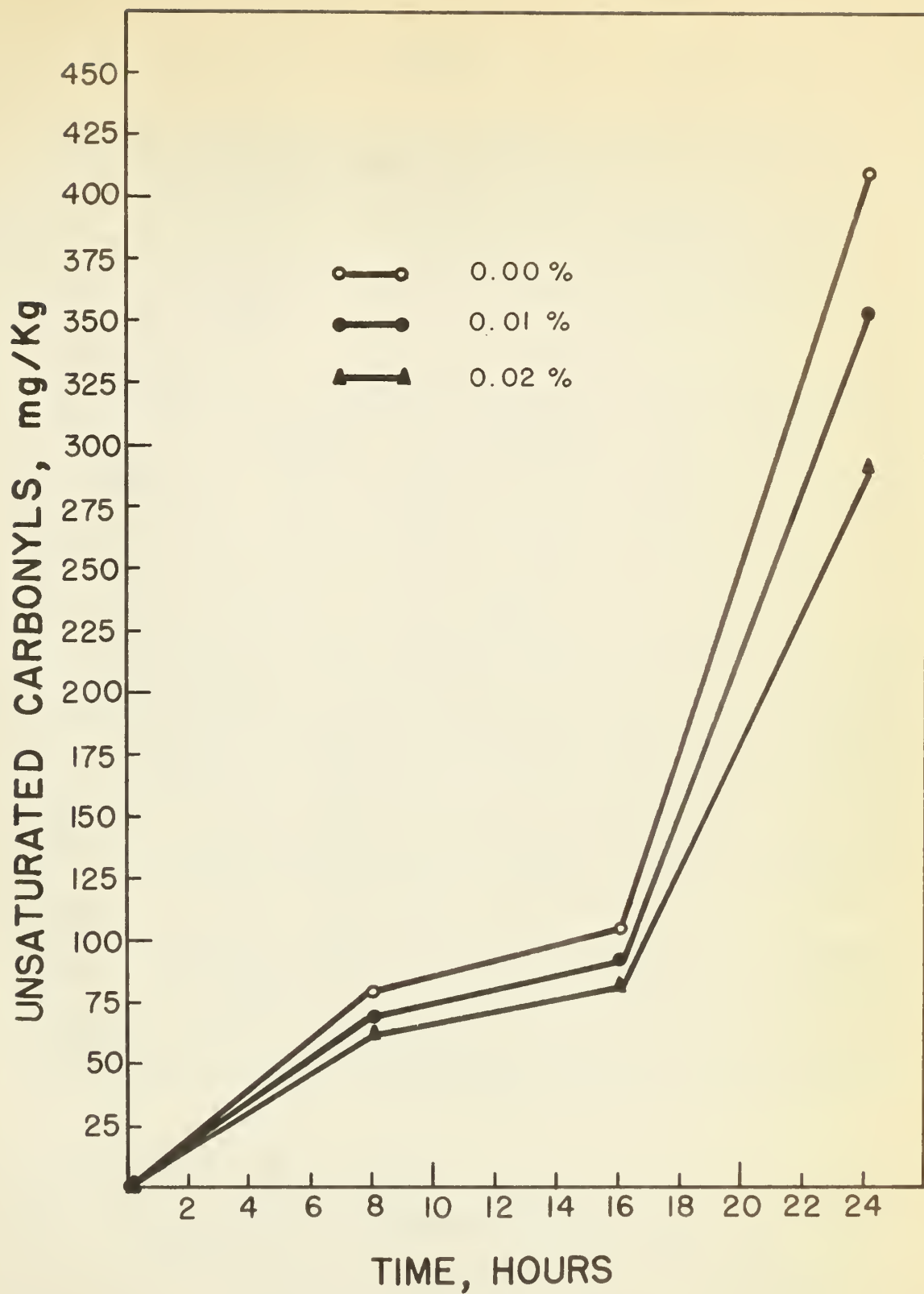


Fig. 10. The effect of ascorbyl palmitate levels on the evolution of volatile unsaturated carbonyls from milk fat at 100°C

Table 22. The half-wave potential^(a) and number of electrons transferred per atom of electroreducible compound of oxidized fats in 1:1 methanol-benzene solution at 25°C^(b)

Order of wave	Cottonseed oil		Milk fat		Lard	
	1		2		3	
	-E _{1/2} , volts	n	-E _{1/2} , volts	n	-E _{1/2} , volts	n
First	0.180 ±.040	1.005 ±.106	0.189 ±.004	0.810 ±.150	0.180 ±.007	1.089 ±.220
Second	0.590 ±.040	0.491 ±.071	0.683 ±.016	0.558 ±.059	0.580 ±.010	0.976 ±.266
Third	1.110 ±.070	0.543 ±.035	1.243 ±.022	0.324 ±.025	1.130 ±.006	0.729 ±.054
Fourth, Mn ⁺⁺	1.420 ±.040	1.451 ±.252	1.430 ±.025	1.106 ±.022	1.450 ±.011	0.904 ±.143

(a) vs. SCE

(b) Cell resistance is 1,240 ohms. Capillary constant, $m^{2/3}t^{1/6} = 2.00, 1.99, 2.00 \text{ mg}^{2/3} \text{ sec}^{-1/2}$ for cottonseed oil, milk fat, and lard respectively.

Table 23. Variation of diffusion current constants in the presence of other electroreducible species

Electroreducible species		C, mM/ lit x 10	$i_d/Cm^{2/3}t^{1/6}$		
			Croton- aldehyde	Hydrogen peroxide	Mangan(II) ions
			1	2	3
Crotonaldehyde	1	4.10	2.42	4.46	
Hydrogen peroxide	2	5.04	2.47	4.90	
Mangan (II) ions	3	4.00			3.54
Crotonaldehyde	4	4.10	2.07	3.86	2.07
Hydrogen peroxide		5.04			
Mangan (II) ions		0.40			
Crotonaldehyde	5	4.10	0.82	3.86	1.67
Hydrogen peroxide		5.04			
Mangan (II) ions		4.00			

Table 24. Variation of the half-wave potentials in the presence of other electroreducible species

Electroreducible species		C, mM/ lit	$-E_{1/2}$ vs. SCE		
			Crotonaldehyde	Hydrogen peroxide	Mangan(II) ions
			1	2	3
Crotonaldehyde	1	4.10	1.43	1.20	
Hydrogen peroxide	2	5.04		1.20	
Mangan (II) ion	3	4.00			1.38
Crotonaldehyde	4	4.10	1.50	1.10	1.50
Hydrogen peroxide		5.04			
Mangan (II) ion		0.40			
Crotonaldehyde	5	4.10	1.38	1.05	1.48
Hydrogen peroxide		5.04			
Mangan (II) ion		4.00			

of the three compounds are shown individually and with respect to the presence of the other two compounds in Tables 23 and 24.

Several authors (49, 44, 72) noticed the occurrence of a prewave in the polarograms of autoxidized fats between 0.00 and -0.10 volts. No particular compound was assigned to this wave. To clarify the nature of this wave the following experiment was conducted. After recording the polarograms of the control milk fat samples (row 8, column 1 of Table 21) 0.5 ml of 0.0126 molar methanolic hydrogen peroxide was added to the solution and the polarograms recorded again. The polarograms of unoxidized milk fat samples or hydrogen peroxide alone (Table 23) did not show the presence of any prewave. However, when the two were polarographed in each other's presence the prewave was distinctly noticeable exactly at the same voltage where the prewave of the oxidized milk fat samples was found, at -0.03 volts (vs. SCE).

Cottonseed Oil

Preparation of samples. Six pounds of refined cottonseed oil were obtained from a commercial source and kept in an airtight container in the dark at -17°C during the time of the experiment.

The copper and iron contents were determined as in the case of milk fat (Table 19). Four lots of samples were prepared; one contained no antioxidant; the others contained 0.01 % ascorbyl

palmitate, 0.01 % cetyl gallate, and 0.02 % cetyl gallate respectively. Further treatment of the lots was the same as for milk fat. The air flow was adjusted to 1.1 liter per minute at 23°C and 766.0 mm Hg pressure per 12 samples. The exit gases from each sample were led through either 10 ml of saturated barium hydroxide solutions or 10 ml of distilled water. The oxidized samples were stored in nitrogen flushed jars, in the dark at -17°C until the analysis took place. The test tubes containing the barium hydroxide solutions were stoppered and stored at room temperature until the estimation of barium carbonate took place. The entire oxidation procedure was repeated twice.

Estimation of carbon dioxide. The saturated barium carbonate-barium hydroxide solutions were centrifuged (1,600 r.p.m., 230 mm radius) for five minutes. The supernatant was poured off. The precipitate was washed six times with 10 ml of distilled water, and finally suspended in five ml of 0.5 normal sulfuric acid. After a few hours of standing, the excess acid was titrated with 0.1 normal sodium hydroxide using methyl red as indicator. The carbon dioxide in grams was calculated from the following equation.

$$\text{g CO}_2/\text{sample} = 44.01 \frac{(V \times N)_{\text{H}_2\text{SO}_4} - (V \times N)_{\text{NaOH}}}{2,000}$$

where:

V = volume of sulfuric acid or sodium
hydroxide in ml

N = normality of the acid or the base

The results obtained are shown in row 3 of Table 25. The figures are lower than the true values by not more than two milligrams; the loss is approximately 5 %, since the solubility of barium carbonate is only 2.4×10^{-3} g per 100 g of water at 24.2°C (32).

Other determinations. All of the following determinations were carried out as described under milk fat. The results of the measurements of the index of refraction, iodometric peroxide values, saturated and unsaturated carbonyls, and 2-TBA numbers are shown in rows 2,4,5,6,7, and 8 respectively of Table 25. The pH determination of the distilled water solutions in the exit gas traps showed no change within ± 0.01 unit. The results of free fatty acid determinations are shown in Fig. 7, and the results of the evaluation of cottonseed oil polarograms in column 1 of Table 22.

Lard

Preparation of samples. Eight pounds of deodorized lard without

Table 25. The effect of two antioxidants on autoxidizing
cottonseed oil at 100°C for 24 hours

Tests		Control	Antioxidant, per cent				
			None	Ascorbyl palmitate	Cetyl gallate		
				0.01	0.01	0.02	
				1	2	3	4
Weight of oxidized sample, gm	1		30.9	30.5	30.3	30.5	
Index of refraction, 50°C n _D	2	1.4619	1.4638				
Carbon dioxide lib- erated in 24 hrs, mg	3		46.3	46.4	46.2	45.9	
Peroxide value, mEq/kg	4	2.8	485.2	478.5	372.4	100.1	
Carbonyls, mM/kg	satura- ted	5	4.4	187.2	77.6	70.1	14.9
	unsatu- rated	6	3.0	161.1	208.9	130.7	13.4
2-TBA numbers, mg/kg	malon- dialde- hyde	7	0.0	3.3	3.6	1.9	0.5
	croton- alde- hyde	8	0.0	672.7	660.2	630.2	133.5

added antioxidant were obtained from a commercial source and stored as described for the other fats.

Polarographic copper and iron contents are shown in Table 19. The oxidation was carried out in the same manner as described under cottonseed oil, and treated similarly to milk fat with the exception that the exit gases were led through either 10 ml of saturated barium hydroxide solutions or the same amount of distilled water. The air flow was set to 1.0 liter per minute at 22°C and 754.0 mm Hg pressure per 12 samples. After autoxidation, the samples and the exit gas trap solutions were handled as described under cottonseed oil. The entire procedure was repeated twice.

Determinations. The results of the measurement of the index of refraction, liberated carbon dioxide, pH of the exit gas trap solutions, iodometric peroxide values, saturated and unsaturated carbonyls, and 2-TBA numbers are shown in rows 2-9 respectively of Table 26. The level of free fatty acids is shown in Fig. 7. The results of the polarography of oxidized lard samples is shown in column 3 of Table 22.

Correlation Coefficients

The results along the rows of Tables 21, 25, 26 show a

Table 26. The effect of two antioxidants on autoxidizing
lard at 100°C for 24 hours

Tests		Control	Antioxidant, per cent				
			None	Ascorbyl palmitate	Cetyl gallate		
				0.01	0.01	0.02	
				3	4	5	
		1	2	3	4	5	
Weight of oxidized sample, gm	1		29.7	29.8	30.0	30.1	
Index of refraction, $n_D^{50^\circ\text{C}}$	2	1.4560	1.4580				
Carbon dioxide liberated in 24 hours, mg	3		24.0	22.8	1.9	1.7	
pH of exit gas trap solutions, 25 ml volumes	4	5.6	2.6	2.6	2.8	4.0	
Peroxide value, mEq/kg	5	5.4	284.6	319.4	70.3	8.0	
Carbonyls, mM/kg	saturated	6	15.8	164.8	102.9	40.1	12.4
	unsaturated	7	2.4	88.8	103.1	59.0	0.2
2-TBA numbers, mg/kg	malon-dialdehyde	8	0.5	5.1	5.0	2.60	0.5
	croton-aldehyde	9	14.3	407.3	419.6	179.7	56.8

uniform trend with respect to the action of antioxidants. Therefore, it is justified to estimate the degree of association of the results of various tests within a table by the means of correlation coefficients. There is not necessarily a cause and effect relationship between the level of constituents under comparison. However, the magnitude of the correlation coefficients is a definite measure of the efficiency of test used for the characterization of the state of autoxidation. The results of the calculations are summarized in Table 27. Iodometric peroxide value (PV) was chosen as one principal variant because of its wide use in fat oxidation research. In addition, the suitability of the measurement of free fatty acids (FFA) for such purposes was also sought.

DISCUSSION

Antioxidants

The results summarized in Table 21 and in Fig. 7 show that ascorbyl palmitate is an effective antioxidant for milk fat, but cetyl gallate has no effect at all. These observations are in agreement with the findings of Somogyi and Kündi - Hegedűs (82) who oxidized milk fat samples at 115°C . However, they measured the development of peroxides only, which left some doubt about

Table 27. Correlation coefficients between the various tests shown in

Tables 21, 25, 26

Tests	Cottonseed oil ^(a)		Milk fat ^(a)		Lard ^(a)	
	PV, mEq/kg	FFA, % oleic	PV, mEq/kg	FFA, % oleic	PV, mEq/kg	FFA, % oleic
Free fatty acid { FFA }, % oleic	0.94		0.97		0.97	
Malondialdehyde, mg/kg	0.98	0.98	0.99	0.94	0.75	0.99
Unsaturated carbonyls, mM/kg	0.97	0.92	1.00	0.96	0.73	0.96
Total carbonyls, mM/kg		0.97		1.00		1.00

(a) All coefficients are highly significant at the 0.01 % level in a t-distribution.

the validity of their findings. On the other hand, the claim of Tollenaar and Vos that gallates are effective antioxidants only for animal fats but not for vegetable oils (91) is in direct conflict with the results shown in columns 2,4,5 of Table 25. Although the authors based their generalization on the observation of the oxidative stability of a number of vegetable oils at 34 and 100°C in the presence of 0.01 % octyl gallate, their list did not include cottonseed oil. They too used only peroxide values for their stability checks.

No tests were run to establish whether the antioxidant concentration during the accelerated tests remained constant. The work of Lundberg et al. (55) indicates that only a small fraction of the antioxidant is destroyed during the first 24 hours in autoxidizing lard at 100°C. It is assumed, therefore, that the progress of autooxidation can be described as if it were under the influence of the initial amount of antioxidant. It is unlikely that the reversed trend of the effectiveness of ascorbyl palmitate and cetyl gallate in milk fat as compared with cottonseed oil and lard is caused by a difference in thermal stability of the antioxidants. The origin of antioxidant specificity must be connected with the variation in fatty acid composition and the state of the metal catalysts during autoxidation. It is peculiar that even after

thorough washing of butter, the milk fat still contained significant amounts of copper and iron (Table 19). Consequently, these metals must be bound to hydrophobic molecules or adsorbed on such entities. In either case they are not present in ionic form. These findings are in agreement with those of Uri (92) who demonstrated the presence of copper, iron, and nickel in samples of highly purified linoleic acid. The initial level of free fatty acids in fats (Fig. 7) is high enough to form salts with the contaminating metals. The significance of heavy metal salts as catalysts of fat oxidation has definitely been established (30). In contrast to this, the role of fatty acid composition cannot be ascertained with clarity. Since the conditions of accelerated oxidations were nearly identical, the greater number of unusual fatty acids in milk fat, as compared with cottonseed oil and lard, (Table 20) must be held responsible for the observed specificity of antioxidants. Olcott has demonstrated (64) that the level of free fatty acids has a definite control over the effectiveness of an antioxidant. He ascribed this role to the liberated protons instead of the anions. The results in Fig. 7 and Tables 21, 25, 26 show that there is indeed a close relationship between antioxidant efficiency and free fatty acid levels. Unfortunately, the independent variable cannot be identified.

Determinations

Index of refraction. During the autoxidation of milk fat the index of refraction changes only to a small extent. Contrary to this, in cottonseed oil, and especially in lard there is a definite increase. According to Chirgwin (17) the rate of change of the refractive index is so rapid at the end of the induction period that it serves as an end-point indicator in the Active Oxygen Method. Although this seems to be possible for lard and cottonseed oil, it is definitely not applicable to milk fat. The reason for the increase of the refractive index is not known. According to Johnson and Kummerow (38) the change is due to the formation of various polymers. Judging from the work of Vandenheuvel and Farmer (94) the reason would seem to be an increase in polyunsaturation. Chirgwin (17) concluded that the change is caused by the formation of large amounts of free radicals and peroxides. The nature of this experiment is such that no valid conclusions can be drawn with respect to the above contradicting views, with one exception. The disappearance of polyunsaturated fatty acids in autoxidizing milk fat (rows 2,3 of Table 21) is accompanied by a small but definite increase in the index of refraction. Therefore, the findings of Vandenheuvel and Farmer (94) are not applicable to autoxidized milk fat.

Peroxides, carbonyls, and 2-TBA values. The positive and significant correlation coefficients between iodometric peroxide values and unsaturated carbonyls (Table 27) show that the two types of compounds are related products of autoxidation. However, the low correlation coefficient between the peroxides and unsaturated carbonyls of lard, together with the results presented in Chapter V show that in certain cases one of either peroxide values or carbonyls cannot be taken as the measure of the other. The peroxide value-malondialdehyde relationship shows the same trend.

It is peculiar that polarography did not reveal the presence of any electroreducible peroxides in the control milk fat samples (column 1 of Table 21), until iodometric peroxide determinations yielded a peroxide value of 13.4. This suggests that during a certain phase of milk fat oxidation there are only cyclic peroxides present but no hydroperoxides or dialkyl peroxides. Direct addition of oxygen to the double bonds forming cyclic peroxides is known to occur (73). The stability of such compounds, however, is thought to be very low (11). In contrast to this, Farmer (24) showed that the initial products of autoxidation are hydroperoxides. The polarographic peroxide values for milk fat are much higher than the corresponding iodometric ones (rows 7,8 of Table 21).

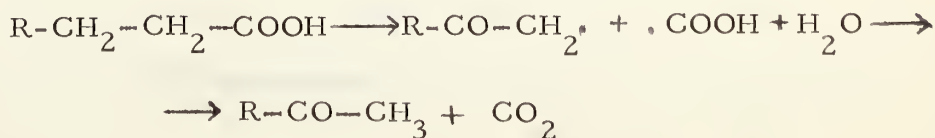
Iodide reacts with all types of peroxides, but only hydroperoxides and certain dialkyl peroxides are reduced at the dropping mercury electrode (98). The iodometric peroxide values, therefore, should be higher than the polarographic ones. There are two systematic sources of error in polarographic peroxide values. It was found (98) that the half-wave potentials of aliphatic diketones overlap that of the hydroperoxides, and according to the results shown in Table 24 the same can happen between 2-unsaturated aldehydes and mangan(II) ions. The relative proportion of these two errors has not yet been established. The results shown in Fig. 8 can be taken only to measure the electroreducible compounds present in milk fat that are reduced at the hydroperoxide and mangan(II) half-wave potentials but not strictly peroxides. The results in Fig. 8 show that the accumulation of such entities is greatly increased after the 16th hour. The same trend is shown in Figures 9 and 10 representing malondialdehyde and volatile unsaturated carbonyls. The length of the induction period for autoxidizing milk fat at 100°C must be around 16 hours. This was also indicated by the bleaching of the samples at that time, and these findings are in agreement with those of Briggs (13).

The saturated to unsaturated carbonyl ratio, as determined by the 2,4-dinitrophenylhydrazine method, is greater than one

for milk fat and lard at all antioxidant levels. The ratio is smaller than one for cottonseed oil in the presence of antioxidants. This is the expected result because of the higher unsaturation of cottonseed oil and because of the greater influence of antioxidants on the oxidation of oleates as compared to linoleates or linolenates (91). As far as steam distillable carbonyls are concerned, the amount of unsaturated carbonyls far exceeds the amount of malondialdehyde and acetaldehyde for all three fats as indicated by the 2-TBA numbers. The difficulty involved in the demonstration of the presence of malondialdehyde in autoxidized fats, as referred to in Chapter III, might well be attributed to the low levels of malondialdehyde.

Acidity. Only a small fraction of the free fatty acids produced during the autoxidation of milk fat and lard are volatile. The significant pH changes of the exit gas trap solutions show that these acids are of short chain, $C_1 - C_3$, having a dissociation constant of 10^{-5} or greater at room temperature. They may include formic, acetic, propionic, oxalic, and malonic acids. Since these acids are not normal constituents of the glycerides of milk fat and lard, they must be produced during the course of autoxidation. At about the time of the completion of these experiments, Loury and Lechartier reported similar results (54). They

froze out the vapours from the exit gases issuing from oleic acid autoxidizing at 80°C. The composition of the water soluble acids collected at -20 to -30°C was found to be 66.7 % formic, 20.6 % acetic, and 12.7 % propionic acid. Up to this time the importance of these fatty acids has not been mentioned. According to the results shown in row 4 of Table 26 and row 6 of Table 21, the amount of volatile, short chain acids is influenced by the presence of antioxidants which is another proof of their autoxidative origin. Loury and Lechartier also observed the development of carbon dioxide. The results shown in row 3 of Table 25 indicate that antioxidants have no effect on the liberation of carbon dioxide from autoxidizing cottonseed oil. On the other hand, cetyl gallate greatly diminishes carbon dioxide formation from lard (row 3 of Table 26). To contrast the different behaviour of the autoxidized fats, it can be recalled that cottonseed oil yielded no volatile short chain acids, and from milk fat no carbon dioxide was liberated. No known mechanisms of autoxidation can account for these differences. According to Loury and Lechartier (54) the following reaction is responsible for the formation of formic acid, carbon dioxide, and methyl ketones.



The larger portion of the free fatty acids produced, however, is not volatile. Endres et al. (22) found that these acids arise by the hydrolysis of triglycerides during thermal oxidation. If this is so, then according to the known mechanisms of autoxidation antioxidants should have no effect on the level of free fatty acids; and yet just the opposite is demonstrated by the results in Fig. 7. The high correlation coefficients between free fatty acid levels and other products of autoxidation are further proof that the formation of free fatty acids is an integral part of the autoxidation mechanism. Although fat autoxidations are known to proceed mainly through a free radical mechanism, the possibility of ionic reactions, such as the formation of free fatty acids, should not be excluded. From the nearly perfect correlation between free fatty acids and total carbonyls one can conclude that in the absence of lipase activity, which is always the case during accelerated tests above 70°C, the change of free fatty acid level is a better criterion of oxidative stability than peroxide values. Therefore, it is suggested that during the Active Oxygen Method of assessing fat stability, a pre-determined free fatty acid level should be chosen as the end point instead of the customary peroxide value.

Polarography

The half-wave potentials of the electroreducible constituents

of cottonseed oil, milk fat, and lard (Table 22) are in close agreement with the exception of the second and third waves of milk fat, which occur at more negative potentials than the others. This suggests that autoxidized milk fat contains more dialkyl peroxides than cottonseed oil or lard. Although the number of electrons transferred per electroreducible molecule is considerably different from small integers, this does not necessarily mean that the electrode processes are irreversible (95). In an electron transfer chain several molecules can share two protons and two electrons. Willits et al. (98) found that the diffusion currents were proportional to the concentration of certain diketones, aldehydes, peroxides, and most hydroperoxides. The possibility of overlapping of half-waves in autoxidized fats, and the change of the diffusion current constants (Tables 24 and 23) indicate that the diffusion current-concentration relationship can only be applied with reservations.

There is considerable difference of opinion about the identity of the various compounds that are assigned to the observed half-wave potentials, although the polarograms are recorded under nearly identical conditions (49, 44). Willits et al. (98) observed that pure hydroperoxides were reduced between -0.61 and -0.96 volts, dialkyl peroxides between 0.00 and -0.19, and 2-unsaturated carbonyls at potentials more negative than -1.5 volts. These

values are reasonably close to those shown in Tables 22 and 24. Kuta and Quackenbush (44) found that the polarographically reducible substances of autoxidized lard showed three waves at -0.15, -0.68, and -1.10, all in good agreement with those shown in Table 22. They did not assign the -0.15 and -0.68 volt waves to any structure, but concluded that the -1.10 volt wave corresponds to hydroperoxides. Ricciuti et al. (72) concluded that all fat hydroperoxides are reduced between -0.69 and -0.96 volts, but they assigned the prewave at -0.10 volts to some unknown peroxidic forms. From the polarographic behaviour of milk fat in the presence of hydrogen peroxide, it can be inferred that the prewave is due to peracids. This view is supported by the found oxidation-reduction potentials of peracids (78). According to the work of Müller (62), on the other hand, one can assume that the prewave is caused by the adsorption of reduced peroxides on the electrode surface.

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